

Production of retinoic acid by antigen presenting cells in the healthy and inflamed human intestine

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Abstract

Murine small intestinal CD103⁺ dendritic cells (DCs) produce retinoic acid (RA) through retinaldehyde dehydrogenase (RALDH) activity, thereby inducing 'gut-homing' $\alpha_4\beta_7$ and CCR9 on T cells they activate, enhancing TGF- β -mediated induction of Foxp3⁺ regulatory T cells and suppressing induction of pro-inflammatory T_H17 cells. RALDH activity of CD103⁺ DCs is reduced in mouse models of inflammatory bowel disease (IBD) but the role of RALDH activity in human intestinal DCs in the pathogenesis of IBD is undefined. This project aimed to determine the influence of inflammation on RALDH activity of antigen presenting cell (APC) subsets including CD103⁺ DCs within human distal intestinal mucosa. RALDH activity was identified by Aldefluor assay in intestinal DCs (CD103⁺ and CD103⁻ subsets) alongside *ALDH1A2* expression in healthy controls. In contrast with mouse models, RALDH activity was not reduced in CD103⁺ DCs from IBD patients. An increased frequency of CD14⁺ macrophages (M Φ) of IBD patients displayed *ALDH1A1*-associated RALDH activity compared with healthy controls. Blood CD14⁺ monocytes, putative precursors of intestinal CD14⁺ M Φ , of healthy controls and IBD patients displayed *ALDH1A1*-associated RALDH activity indicating RALDH is systemically acquired by monocytes and upregulated within the mucosa of IBD patients, or alternatively that RALDH⁺ monocytes are selectively recruited in IBD. *In vitro*, inhibition of RA receptor- α signalling blocked GM-CSF-mediated differentiation of TNF α -producing pro-inflammatory RALDH⁺ CD14⁺ M Φ from monocytes, consistent with enhanced RALDH activity of intestinal CD14⁺ M Φ in IBD supporting a pro-inflammatory phenotype. Soluble intestinal mediators including prostaglandin E₂ suppressed RALDH activity of MoDCs *in vitro*, whilst mediators from inflamed IBD mucosa conditioned MoDCs to imprint enhanced levels of $\alpha_4\beta_7$ expression on naive CD4⁺ T cells independent of RALDH activity. This study provides the first systematic analysis of

RALDH activity in human intestinal APCs and indicates important distinctions between mouse models and human IBD.

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Abbreviations

ALDH	Aldehyde dehydrogenase
APC	Antigen presenting cell
BM-DC	Bone marrow-derived dendritic cell
BODIPY	Boron-dipyromethene
CD	Crohn's disease
C/EBP β	CCATT enhancer-binding protein β
CM	Conditioned medium
CRBP	Cellular retinol binding protein
DC	Dendritic cell
DEAB	Diethylaminobenzaldehyde
DMSO	Dimethyl sulphoxide
EP	E-prostanoid receptor
ERK	Extracellular signal-regulated kinase
GM-CSF	Granulocyte macrophage colony stimulating factor
HSC	Haematopoietic stem cell
IBD	Inflammatory bowel disease
iNOS	Inducible nitric oxide synthase
JNK	cJun N-terminal kinase
LPS	Lipopolysaccharide
LC/MS/MS	High performance liquid chromatography with tandem mass spectrometry

MΦ	Macrophage
MAPK	Mitogen associated protein kinase
MLN	Mesenteric lymph node
MoDC	Monocyte-derived dendritic cell
NF-κB	Nuclear factor κB
PBMCs	Peripheral blood mononuclear cells
PGE ₂	Prostaglandin E ₂
PP	Peyer's patch
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SD	Standard deviation
SI-LP	Small intestinal lamina propria
TLR	Toll-like receptor
UC	Ulcerative colitis

Chapter 1: Introduction

1:1 Overview

The intestine must perform two basic functions critical for survival: release and uptake of nutrients from ingested material and the immunological defence of a large internal surface area. This second component is complicated by the fact that the intestine is colonized by a complex and abundant microbiota comprising bacteria, viruses, fungi and parasites. In general, the intestinal microbiota benefit the host e.g. through the break down and release of nutrients from otherwise non-digestible food molecules, and the restriction space for the access of pathogens (Backhed et al., 2005, Abraham and Medzhitov, 2011). Overall therefore, the intestinal immune system must exist in a balanced state between pro-inflammatory activity induced by pathogens entering the intestinal mucosa, and low or non-responsiveness to the microbiota and particles derived from the digestion of food. This careful balance in activities is disturbed in inflammatory bowel disease (IBD), where an inappropriate immune response is believed to propagate against microbiota-derived antigen in genetically susceptible individuals, resulting in a state of chronic inflammation, tissue damage and functional failure of the intestine (reviewed by Baumgart and Carding, 2007). The pathogenesis of IBD however remains incompletely understood.

The questions central to this thesis relate to the role of a specific group of immune cells, the mononuclear phagocytes (macrophages and dendritic cells), in the pathogenesis of IBD in humans. In particular, this work seeks to determine the contribution of the vitamin A metabolite retinoic acid (RA) in the activity of these cells in the healthy and inflamed human intestine, which is involved in the intestinal immune system of mice through modulation of the intestinal adaptive response (see section

1:8), induction of lymphocyte gut-homing (see section 1:10) and the induction of secretory IgA production by B cells (see section 1:8.6).

This introduction will provide a broad overview of the known activities of various dendritic cell and macrophage populations in the murine and human intestine and how these functions are influenced by RA. Attention will then turn to understanding contribution of these subsets to the pathogenesis of IBD in humans and experimental models of intestinal inflammation in mice. Finally, this introduction will finish with a discussion of the principal aims and hypothesis of this thesis.

1:2 Anatomy of the intestine

1:2.1 Small intestine

The small and large intestine are distinguished by function and morphology. The small intestine is the principal site of food digestion and absorption. Beginning as the duodenum in direct contact with the pyloric sphincter, it is further sequentially divided into the jejunum and ileum, of which the most distal part is the terminal ileum (TI). The exterior surface of the small intestine is the serosa (visceral peritoneum), beneath which is the subserosa and then a sequentially longitudinal and circular muscle layer (muscularis externa). This is followed by the loose, moderately vascular connective tissue of the submucosa which contains blood vessels, lymphatics and nerves. A thin layer of longitudinal and circular smooth muscle fibres (muscularis mucosae) separates the submucosa from the mucosa, comprising the loose connective tissue of the lamina propria (LP) and a single layer of epithelial cells (reviewed by Moore et al., 2006, Day and Morson, 2003). The small intestinal mucosa is characterised by the presence of villi, finger-like structures that project into the intestinal lumen and significantly increase

the absorptive surface area (see Figure 1.1). There are between 10 and 40 villi per mm^2 of mucosa, separated by crypts which extend down to the muscularis mucosae. The epithelium is continuous between crypt and villus, and is continually replenished by stem and committed progenitor cells towards the crypt base reviewed by Day and Morson, 2003).

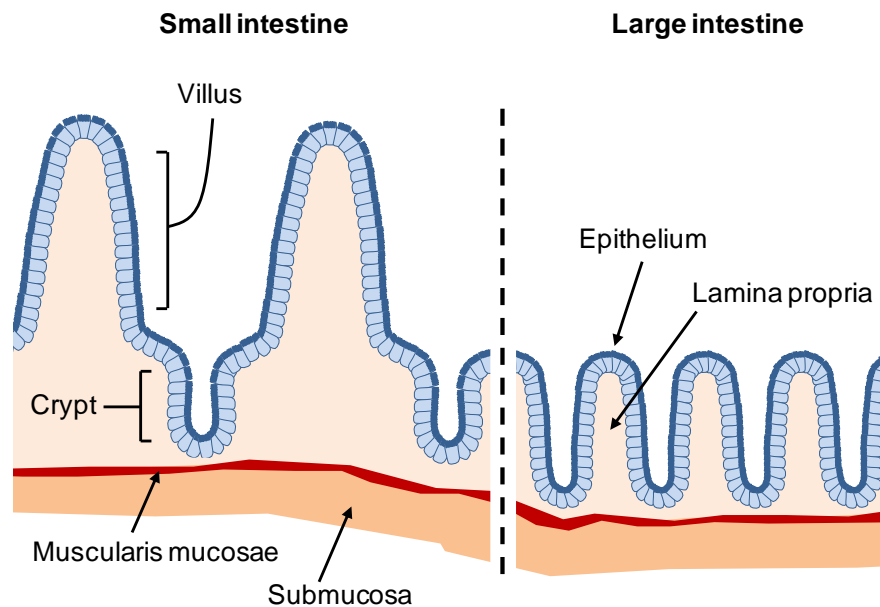


Figure 1.1: Structure of intestinal mucosa. Mucosa contains the surface epithelium and underlying connective tissue of the lamina propria. Small intestinal mucosa is arranged into finger-like villi projections separated by crypts containing stem cells which continually replenish the epithelium. Large intestinal mucosa is devoid of villi but contains crypts. The mucosa is separated from the submucosa by a thin layer of smooth muscle cells, the muscularis mucosae. Beneath the submucosa is the muscularis externa and serosa (not shown).

1:2.2 Large intestine

The large intestine is primarily involved in absorption of water and electrolytes from indigestible material passed from the small intestine. It begins at the caecum which connects directly with the TI, and is also linked to the closed tubular structure of the appendix. From the caecum, the large intestine is divided into ascending, transverse,

descending and sigmoid colon, before ending at the rectum and anal canal. The arrangement of tissue layers is similar to the small intestine, though the longitudinal muscularis externa layer is narrow and shorter than the length of the large intestine, causing it to bunch into distinctive haustral clefts. Whilst the mucosa of the large intestine is devoid of villi, the epithelium is continuously replenished by crypt stem and committed progenitor cells as in the small intestine (reviewed by Day and Morson, 2003).

1:2.3 Gut-associated lymphoid tissue

The gut associated lymphoid tissue (GALT) includes isolated lymphoid follicles (ILFs) which are regions of the duodenal and jejunal mucosa and/or submucosa 0.6 to 3.0 mm in size (in humans) containing a high concentration of lymphocytes arranged into B cell follicles with T cell zones in the interfollicular areas, as well as macrophages (MΦ) and dendritic cells (DCs). ILFs aggregate in the distal ileum to form Peyer's patches (PPs) up to 12 x 20 mm in size in humans (reviewed by Day and Morson, 2003). The follicle associated epithelium (FAE) overlying ILFs and PPs contains specialized epithelial microfold (M) cells which lack the brush border and mucus coverage of conventional enterocytes (reviewed by Mowat, 2003). M cells are highly specialized for phagocytosis and transcytosis of commensal and pathogenic bacteria as well as particulate antigen and macromolecules (reviewed by Mabbott et al., 2013). DCs in the extracellular space immediately below M cells collect antigen from these cells and present it directly to naive B cells inducing the formation of IgA⁺ plasma cells (Macpherson and Uhr, 2004) or present the antigen in a processed form to local T cell populations (discussed further in section 1:8). As discussed in section 1:4.4, secretory IgA provides an important component of intestinal barrier function.

1:2.4 Lymphoid tissue draining intestinal mucosa

Extracellular fluid from the intestinal LP and GALT is collected by lymphatic vessels which then drain to a network of lymph nodes. In the human, jejunal and ileal lymphatics sequentially drain into juxta-intestinal lymph nodes close to the intestinal wall, then onto the mesenteric lymph nodes (MLNs), where the mesentery is a fan-shaped fold of the peritoneum that holds the jejunum and ileum to the posterior abdominal wall (reviewed by Moore et al., 2006). MLNs then drain into superior MLNs and then into blood circulation via the thoracic duct. The section between the TI and up the ascending colon drains into ileocolic lymph nodes, whilst the transverse colon drains into middle colic lymph nodes. Both ileo- and middle colic lymph nodes drain into the superior MLN. The descending and sigmoid colon instead drain into the intermediate colic lymph nodes and then onto the inferior MLNs. The rectum and anal canal drain into the iliac nodes, which also drain the pelvic area. Throughout the colon are epicolic (attached to the intestinal wall) and pericolic (close to the intestinal wall) which first collect lymph before passing it on to the respective lymph nodes described above (reviewed by Moore et al., 2006).

1:3 Nature of intestinal antigen

The intestine harbours a complex microbial community of bacteria, fungi, parasites and viruses collectively termed the 'microbiota'. This constitutes more than 100 trillion microorganisms in the human gastrointestinal tract, of which the majority are bacteria and are found in greatest density in the large intestine (reviewed by Kamada et al., 2013). Such bacteria exist in a mutually beneficial symbiotic relationship with the host whereby bacteria are provided with an anaerobic environment and access to large quantities of dietary polysaccharides such as plant-derived pectin, cellulose and

resistant starches as a source of nutrients. In turn, bacteria degrade these otherwise indigestible products releasing nutrients for the benefit of the host (reviewed by Backhed et al., 2005). Symbiotic bacteria also out-compete many pathogenic bacteria for nutrients within the specialized environment of the intestine, as well as limiting the physical space available for colonisation by pathogens (reviewed by Hooper and Macpherson, 2010). The intestinal bacterial population is highly diverse at a species level and varies widely between individuals. The principal phyla however are the gram positive Firmicutes and gram negative Bacteroidetes (reviewed by Hooper and Macpherson, 2010).

A wide range of immune cells as well as non-haematopoietic cells including epithelial cells express both cell surface and intracellular pathogen-associated molecular pattern (PAMP) receptors such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) proteins. Interaction of these receptors with their microbial ligand triggers dramatic changes in cell behaviour including the production of a range of pro-inflammatory cytokines aimed at responding to microbial presence within the tissue (reviewed by (Akira et al., 2006). A key component of intestinal homeostasis is therefore the physical separation of the intestinal microbiota from the interior of the mucosa, so as to restrain uncontrolled inflammatory responses and thus prevent immunopathology.

1:4 Restriction of access of luminal antigen to the intestinal mucosa

1:4.1 Mucus

The luminal epithelial surface of the murine large intestine is covered by two distinct

mucus layers. The inner layer is adherent to the epithelium, densely arranged and devoid of bacteria, whilst the outer layer is more loosely arranged and is penetrated by bacteria (Johansson et al., 2008). The main structural component of intestinal mucus is the heavily glycosylated protein MUC2. Produced by goblet cells, MUC2 forms a gel-like polymeric net structure through disulphide bonds (reviewed in Johansson et al., 2011). Murine small intestinal epithelium is covered by a single mucus layer which is penetrable by bacteria-sized particles (Ermund et al., 2013). The significance of the contrasting nature of mucus in the small and large intestine is not fully understood, but may reflect the more absorptive function of the small intestine and the need to protect the mucosa from the large bacterial burden of the large intestine. Antimicrobial peptides including the defensins HBD-1 and HBD-3, the cathelicidin LL37 and lysozyme are present within human rectal mucus, potentially further restricting bacterial encroachment (Antoni et al., 2013). *Muc2*^{-/-} mice display bacterial encroachment directly onto the large intestinal epithelium (Johansson et al., 2008), associating with the onset of colitic symptoms including diarrhoea, rectal prolapse as well as severe growth retardation (Van der Sluis et al., 2006).

It has been proposed that the pathogenic bacteria *Campylobacter* which causes diarrheal disease in humans is able to penetrate the viscous mucus layer and reach the epithelium due to high motility conferred by flagella (reviewed by Guerry, 2007).

1:4.2 Epithelial barrier

Beneath the mucus layer, the intestinal epithelium acts as a physical barrier between luminal antigen and the interior of the mucosa. It is formed by a single and continuous layer of cells separated from the LP by a basement membrane. Neighbouring cells are held in close association through tight junction proteins such as zonula occludens 1

(ZO-1) and claudins which block the passage of from the apical to basal side of the epithelium (reviewed by Abreu, 2010). Four distinct cell types are found within the epithelium. Enterocytes are the most abundant, columnar in shape with a brush border lined with microvilli that assists in their absorptive function. Goblet cells produce large quantities of mucins which form the mucus layer as described in section 1:4.1 (reviewed by Day and Morson, 2003). Paneth cells produce most of the anti-microbial peptides in the small intestine, including α -defensins, whilst most epithelial cells produce β -defensins (reviewed by Hooper and Macpherson, 2010). Enteroendocrine cells make up approximately 1 % of epithelial cells and produce a wide range of gastrointestinal hormones (Rehfeld, 1998). All four cell types originate from stem cells located within each crypt base. These cells asymmetrically divide and generate committed progenitor cells which push the cells above up the crypt wall. These progenitors then terminally differentiate before reaching the exposed surface epithelium. The continual upward motion of cells replenishes those sloughed off following the transit of luminal material (reviewed by Humphries and Wright, 2008).

1:4.3 Antimicrobial peptides

As discussed, the intestinal epithelium also generates a range of antimicrobial peptides including defensins, cathelicidins, and C-type lectins which kill bacteria directly through enzymatic attack of the bacterial cell wall or inner membrane (reviewed by Hooper and Macpherson, 2010). Several of the α -defensins are generated constitutively by the murine intestinal epithelium independent of the presence of bacteria (Putsep et al., 2000). In contrast, the presence of bacteria in the intestine induces production of the antibacterial C-type lectin regenerating islet-derived protein 3 γ (REG3 γ) in the murine small intestine (Cash et al., 2006, Brandl et al., 2007, Vaishnava et al., 2008). The vast

majority of antimicrobial activity in the murine small intestine is confined to the mucus layer as opposed to the intestinal lumen (Meyer-Hoffert et al., 2008). Targeted disruption of TLR signalling in murine intestinal epithelial cells through deletion of the MyD88 gene (a key signalling molecule in several TLR signalling pathways) leads to significantly increased levels of mucus-associated bacteria as well as translocation of bacteria to the interior of the mucosa, associated with reduced expression of REG3 γ and MUC-2 (Frantz et al., 2012). Bacterial stimulation through epithelial TLRs also induces the production of factors which protect the intestinal epithelium from injury, including IL-6 (Rakoff-Nahoum et al., 2004). TLR2 signalling in epithelial cells helps to maintain epithelial integrity in mice during infection with *Citrobacter rodentium* through enhanced expression of the tight junction protein ZO-1 (Gibson et al., 2008). Overall, this demonstrates that in addition to acting as a physical barrier, the intestinal epithelium is able to sense and respond to bacterial encroachment directly.

1:4.4 Secretory IgA

Intestinal barrier function is further supported by secretory IgA (sIgA) produced by plasma cells (terminally differentiated B cells) within the LP. Expression of the J chain by plasma cells allows the formation of dimeric and larger polymers of IgA and facilitates transport of sIgA across the epithelium by polymeric Ig receptor (pIgR)-mediated transcytosis (reviewed by Macpherson et al., 2008). sIgA in the intestinal lumen helps to limit bacterial association with the epithelial surface (Suzuki et al., 2004). The polymeric nature of sIgA is likely to increase the avidity of antigen binding and promote agglutination of intestinal bacteria (reviewed by Macpherson et al., 2008). The induction of sIgA is discussed in greater detail in section 1:8.6. The overall features of the intestinal barrier are summarised in Figure 1.2.

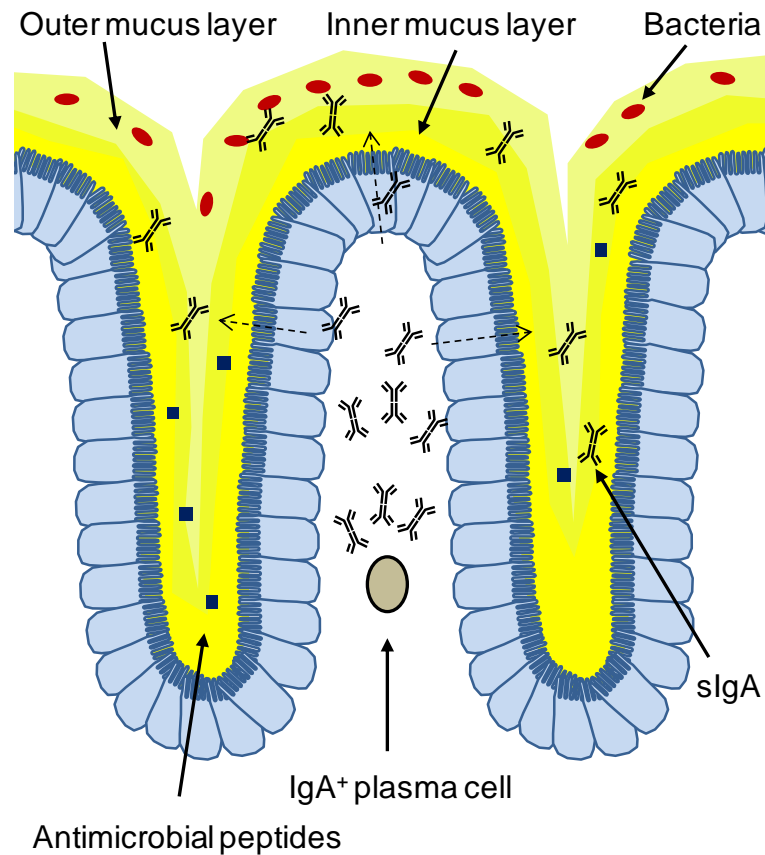


Figure 1.2: Barrier function of the epithelium in the large intestine. The inner mucus layer adjacent to the epithelium is densely packed and largely devoid of bacteria in the steady state. The outer layer is more loosely arranged and contains a large quantity of bacteria. Secretory IgA (sIgA) produced by IgA⁺ plasma cells in the LP is passed through epithelial cells by pIgR-mediated transcytosis and agglutinates bacteria, preventing encroachment. Antimicrobial peptides including defensins produced by epithelial cells further prevent bacterial encroachment into the crypts.

1:5 Intestinal macrophages

Whilst the combined action of the epithelial barrier and a mucus layer embedded with anti-microbial peptides and secretory IgA restricts access of the intestinal microbiota to the apical surface of the intestinal epithelium and the interior of the mucosa, the entry of microorganisms into the mucosa cannot be prevented entirely. M cells in the epithelium

overlying gut-associated lymphoid (GALT) tissue promote bacterial encroachment through diminished mucus coverage and brush border, facilitating direct uptake of antigen including live bacteria by transcytosis into the interior of the tissue (reviewed by Mowat, 2003). Many bacterial pathogens including *Salmonella*, *Yersinia* and *Shigella* sub-species exploit M cells as an entry point into the intestinal mucosa (reviewed by Mowat, 2003). Bacteria may also enter the intestinal mucosa through damage to the intestinal epithelium. The intestinal immune system needs to rapidly clear bacterial presence from within the mucosa in order to prevent damaging chronic inflammatory responses. This section will focus on the distinct roles of human and murine mononuclear phagocytes in helping to maintain intestinal homeostasis.

1:5.1 Human intestinal resident macrophages

The human intestinal mucosa contains a large population of resident macrophages (MΦ), members of the mononuclear phagocyte group, which efficiently phagocytose and kill bacteria including *Escherichia coli* and *Salmonella typhimurium* but do so without producing pro-inflammatory cytokines such as TNFα, IL-1β or IL-6 (Smythies et al., 2005). The majority of human intestinal resident MΦ do not produce reactive oxygen species (ROS) such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), indicating that this killing mechanism is independent of ROS (Mahida et al., 1989).

The low responsiveness of human intestinal MΦs to microbial stimulation stems in part from lack of expression of a range of microbial ligand receptors including the lipopolysaccharide (LPS) co-receptor CD14 (Smythies et al., 2005, Schenk et al., 2005). The LPS receptor TLR4 is also expressed at low levels by intestinal resident MΦ when compared with non-intestinal cells such as circulating CD14⁺ monocytes (Smythies et al., 2010). An additional receptor which is expressed at high levels on

blood CD14⁺ monocytes but is very low or absent on human intestinal resident MΦ is triggering receptor expressed on myeloid cells (TREM)-1 (Schenk et al., 2005). Whilst the physiological ligand of this receptor is unknown, stimulation of monocytes with TREM-1 agonistic antibodies synergistically enhances production of pro-inflammatory cytokines including TNFα and IL-1β in response to bacterial stimulation (Bouchon et al., 2001).

TLR3 recognises double stranded (ds)RNA which is an indicator of viral infection (Alexopoulou et al., 2001). Despite expressing TLR3, intestinal resident MΦ do not respond to stimulation with the synthetic dsRNA molecule poly I:C (Smythies et al., 2010). This indicates that the non-responsiveness of these cells to microbial stimulation extends beyond low expression of pattern recognition receptors (PRRs). A key downstream signalling consequence of the stimulation of TLRs is activation of the transcription factor NF-κB. In the steady state, NF-κB is retained in the cytosol due to an association with the protein IκBα. Signalling through TLRs leads to phosphorylation and degradation of IκBα, freeing NF-κB to translocate into the nucleus where it can direct the transcription of pro-inflammatory cytokines including TNFα (reviewed by Baldwin, 1996). The low inflammatory activity of human resident intestinal MΦ is determined in part by a reduced ability to phosphorylate IκBα in response to microbial stimulation, leading to defective NF-κB signalling (Smythies et al., 2010). Furthermore, reduced expression of the TLR signalling adaptor proteins MyD88 and TRIF may also contribute to the weak responses of human intestinal resident MΦ to microbial stimulation (Smythies et al., 2010).

A small population of CD14⁺ MΦ exists within the steady state human intestinal mucosa which is able to produce a range of pro-inflammatory cytokines including TNFα and IL-6 in response to stimulation *in vitro* with both heat-killed *E. coli* and *E. faecalis* (Kamada

et al., 2008). The low numbers of these cells in comparison with intestinal resident MΦ (which are CD14⁺) suggests that the overall response to bacterial encroachment into the human intestinal mucosa in the steady state is clearance by phagocytosis and intracellular killing without the induction of chronic inflammation.

1:5.2 Murine intestinal resident macrophages

Human intestinal resident MΦ are marked by an absence of either pro or anti-inflammatory cytokine production upon microbial stimulation (Smythies et al., 2005). In contrast, murine intestinal mucosa contains a large population of resident MΦ that respond to microbial stimulation with production of the anti-inflammatory cytokine IL-10, as well as producing IL-10 constitutively (Denning et al., 2007, Bain et al., 2013, Rivollier et al., 2012). Moreover, murine resident intestinal MΦ in the large intestinal mucosa do not express TLRs and do not produce pro-inflammatory cytokines in response to microbial stimulation (Platt et al., 2010).

CX₃CR1⁺ mononuclear phagocytes extend cellular protrusions through the intestinal epithelium of the murine terminal ileum (TI) (Niess et al., 2005, Rescigno et al., 2001). Demonstration of this activity requires *Cx3cr1^{+/gfp}* mice in which one allele of the *Cx3cr1* gene is replaced with green fluorescent protein (GFP), allowing the direct visualization of CX₃CR1-GFP^{hi} cells (Jung et al., 2000). At this time, CX₃CR1-GFP^{hi} cells were identified as dendritic cells (DCs; see section 1:6) on the basis of a CD11c⁺ MHC II⁺ phenotype, and the activity was proposed as a method of sampling antigen directly from the intestinal lumen (Rescigno et al., 2001). However, more recent studies have determined that murine intestinal CX₃CR1-GFP^{hi} MΦ in the steady state possess MΦ as opposed to DC features, including a lack of migration from the LP in draining lymphatics, slow turnover, vacuolar morphology, high phagocytic activity (compared

with DCs) and low stimulatory capacity for naive T cells (Schulz et al., 2009, Rivollier et al., 2012). They are identified as F4/80^{hi}, CD11b⁺ and either CD11c⁺ or CD11c⁻ (Bain et al., 2013, Rivollier et al., 2012). These and other phenotypic features of CX₃CR1-GFP^{hi} MΦ are listed in Table 1.1.

Table 1.1: Phenotypic distinction of murine steady state intestinal mononuclear phagocytes

Marker	CD103 ⁺ CD11b ⁺ DCs	CD103 ⁺ CD11b ⁻ DCs	CD103 ⁻ DCs	CX ₃ CR1-GFP ^{hi} MΦ
CD11b	+	-	+	+
CD11c	+	+	+	+ or -
CX ₃ CR1-GFP	-	-	+	+++
MHC class II	+	+	+	+
F4/80	+	-	+	+++
CD172α	+	-	+	+
CD8α	-	+ or -	-	-
CD103	+	+	-	-
CD64	-	-	-	+
Migration	+	+	+	-
Naïve T cell stimulation	+++	+++	+++	+

The phenotypic features listed in the table above derive from the following publications: (Bogunovic et al., 2009, Schulz et al., 2009, Rivollier et al., 2012, Cerovic et al., 2012, Bain et al., 2013, Farache et al., 2013).

The extension of transepithelial protrusions by CX₃CR1-GFP^{hi} MΦ in mice requires the expression of CX₃CR1 (Niess et al., 2005) which is the receptor for CX₃CL1 (Imai et al., 1997). CX₃CL1 (fractalkine), is expressed on the basolateral surface of intestinal epithelial cells in mice and humans (Niess et al., 2005, Muehlhoefer et al., 2000). This process however is mouse strain-dependent, as it is observed in C57BL/6 but not in BALB/c mice (Vallon-Eberhard et al., 2006). The MLNs of *Cx3cr1*^{-/-} mice contain

significantly increased bacterial density as composed to WT mice, and these bacteria are predominantly of commensal origin as members of the Firmicute phyla (Medina-Contreras et al., 2011). This indicates that CX₃CR1-GFP^{hi} MΦ support intestinal homeostasis through bacterial clearance, preventing dissemination of bacteria to the draining MLN. Moreover, CX₃CR1⁺ cells migrate through the small intestinal epithelium of mice upon stimulation with Salmonella, and are present within the intestinal lumen containing intracellular Salmonella (Arques et al., 2009). However, CX₃CR1⁺ cells displaying this activity are CD11b⁻, and enter the intestinal lumen in BALB/c mice (Arques et al., 2009). The extent to which this represents CX₃CR1⁺ MΦ or a CX₃CR1⁺ CD103⁻ DC population (see section 1:6.2) remains controversial.

Efficient bacterial killing is important in maintaining intestinal homeostasis in mice. Two important mechanisms of bacterial killing are the production of reactive oxygen species such as hydrogen peroxide (H₂O₂) and superoxide (O₂⁻) by NADPH oxidase (reviewed by Nathan and Cunningham-Bussel, 2013) and reactive nitrogen intermediates (RNI) including nitric oxide (NO) produced by inducible nitric oxide synthase (NOS2) (reviewed by Nathan and Hibbs, 1991). Mice genetically deficient in both pathways are highly susceptible to infection and the formation of large abdominal abscesses containing bacteria from the commensal microbiota (Shiloh et al., 1999). Peripheral MΦ obtained from these mice have a significantly attenuated ability to kill bacteria *in vitro*, consistent with a role in maintaining control of the intestinal microbiota (Shiloh et al., 1999).

1:6 Intestinal dendritic cells

The abundant population of resident intestinal MΦ in mice and humans is likely to be able to clear non-pathogenic bacteria entering the intestinal mucosa. However,

pathogenic bacteria entering the intestinal mucosa have evolved virulence factors that allow them to evade killing by MΦ, enabling them to cause infection. As an example, pathogenic bacteria of the genus *Yersinia* resist phagocytosis by intestinal MΦ in mice through intracellular injection of the virulence factors YopE, YopH and YopT which inactivate the actin cytoskeleton (reviewed by Sansonetti, 2004). Pathogens are thus able to overwhelm the initial innate line of immune defence in the intestine. In order to contain the infection, the body mounts an adaptive immune response through which an enhanced level of microbial killing is targeted specifically to the antigens associated with infection (discussed in more detail in section 1:8). However, it is important that this adaptive immune response is not applied to harmless antigens such as those derived from food particles, or those associated with the commensal microbiota. The innate and adaptive immune systems are linked in the intestinal mucosa by dendritic cells (DCs).

1:6.1 General features of dendritic cells

DCs exist in peripheral tissues in an immature state characterised by high endocytic activity alongside a low capacity to activate naive T cells (reviewed by Cella et al., 1997). Antigen sampling is performed by different forms of endocytosis including macropinocytosis whereby extracellular fluid containing soluble antigen is constitutively taken up at the cell membrane, and phagocytosis where large antigen including whole bacteria are internalized (Sallusto and Lanzavecchia, 1994). Uptake of antigen is facilitated by expression of a range of surface receptors including FcγRII which allows DCs to interact with and take up IgG-antigen immunocomplexes, and the mannose receptor which facilitates interaction with mannosylated antigen (Sallusto et al., 1995).

Internalized antigen is degraded to constituent peptides within lysosomal compartments and the peptides are loaded on MHC class II molecules and presented at the cell

surface. Whilst in an immature state, MHC class II molecules undergo constitutive internalisation and recycling to endosomal compartments (Pinet et al., 1995).

Antigen internalized by phagocytosis and macropinocytosis may also be passed into the cytosol, processed by the proteasome and derived peptides presented at the cell surface in conjunction with MHC class I (Kovacsovics-Bankowski and Rock, 1995, Norbury et al., 1995, Reis e Sousa and Germain, 1995). This 'cross presentation' pathway of antigen presentation is important in that MHC class I is used by all nucleated cells (i.e. including non-professional antigen presenting cells) primarily to present cytosol-derived peptides e.g. deriving from a viral infection (reviewed by Cella et al., 1997). Presentation of exogenously derived antigen by DCs in association with MHC class I allows priming of CD8⁺ cytotoxic T cells (discussed further in section 1:8.4).

Upon stimulation with inflammatory mediators including TNF α , IL-1 β or LPS, DCs undergo CCR7-dependent migration to the T cell zones of draining lymph nodes as well as a process of maturation (reviewed by Alvarez et al., 2008). Maturation entails a simultaneous reduction in the endocytic capacity of cells and increased surface MHC expression through reduced MHC internalization (Pierre et al., 1997 and reviewed by Cella et al., 1997) alongside increased expression of co-stimulatory molecules such as CD80 and CD86 which interact with CD28 on naive T cells and promote T cell activation (reviewed by Sharpe and Freeman, 2002). Overall, this process renders DCs able to efficiently activate naive CD4⁺ and CD8⁺ T cells which express TCRs specific for the presented antigen. The distinct profile of cytokines produced during interaction of DCs and T cells also shapes the particular profile adopted by proliferating T cells (discussed further in section 1:8).

1:6.2 Murine intestinal dendritic cells

Multiple DC subsets are present within murine intestinal mucosa. DCs expressing CD103 (α_E integrin which forms a heterodimer with β_7) are present in murine LP in close association with the epithelium of both the small and large intestine, and includes both CD11b⁺ and CD11b⁻ CD8 α ⁺ subsets (Rivollier et al., 2012, Farache et al., 2013, Bogunovic et al., 2009). CD103⁺ CD11b⁻ CD8 α ⁺ DCs are also present in greater concentration in murine PPs as compared with murine SI-LP (Bogunovic et al., 2009).

The presence of luminal *Salmonella typhimurium* draws murine SI-LP CD103⁺ DCs to the SI epithelium whereupon they extend dendrites between epithelial cells in order to take up bacteria by phagocytosis before returning to the LP (Farache et al., 2013). It has also recently been proposed that goblet cells provide a passage for low molecular weight soluble antigen e.g. ovalbumin through to the interior of mouse and human SI-LP (goblet cell-associated antigen passages, or GAPs) for collection, at least in the mouse, by CD103⁺ DCs associated with the basolateral GAP surface (McDole et al., 2012). Antigen sampling by murine CD103⁺ DCs in the small intestinal mucosa is summarised in Figure 1.3.

CD103⁻ DCs are also found within the large intestinal LP of mice (Rivollier et al., 2012), although it is unclear whether these cells can also pass dendrites through the epithelium to sample luminal antigen. The ability of DCs to interact with the intestinal epithelium in response to *Salmonella typhimurium* is unaffected in *Itgae*^{-/-} mice (deficient in CD103) (Diehl et al., 2013), indicating that expression of CD103 is not required for this process. The lack of CD103 expression by CD103⁻ DCs would therefore not preclude *Salmonella*-induced sampling of luminal antigen closely associated with the epithelium by this subset.

The identification of murine conventional CD103⁻ DCs is made difficult by the fact that historically DCs were identified as CD11c⁺ MHC II⁺ cells. Murine intestinal resident CX₃CR1-GFP^{hi} MΦ express MHC II and a significant fraction are CD11c⁺ (Schulz et al., 2009, Rivollier et al., 2012, Bain et al., 2013). Therefore, murine studies referring to CD103⁻ DCs may include CX₃CR1⁺ MΦ. To distinguish these groups, CD103⁻ DCs express low to intermediate levels of F4/80, whilst CX₃CR1-GFP^{hi} MΦ are F4/80^{hi} (Schulz et al., 2009, Rivollier et al., 2012, Bain et al., 2013). CD103⁻ DCs are also CD11b⁺, and are CX₃CR1-GFP^{int} in *Cx3cr1^{+/gfp}* mice (Rivollier et al., 2012). Phenotypic distinction of these subsets is outlined in Table 1.1.

In addition to sampling luminal antigen directly, murine intestinal DCs also phagocytose apoptotic epithelial cells before migration into the draining MLNs. This behaviour is attributed to CD11c⁺ CD11b⁺ and CD11c⁺ CD11b⁻ CD8α⁺ cells (Jang et al., 2006). Whilst the CD11b subset may in fact contain both DCs and CX₃CR1-GFP^{hi} MΦ, the CD11b⁻ CD8α⁺ subset is likely to be entirely DCs as resident MΦ are CD11b⁺ in the murine intestine (Bain et al., 2013, Rivollier et al., 2012).

Expression of CCR7 allows murine intestinal CD103⁺ DCs to migrate from the SI-LP to the T cell zones of draining MLNs along a gradient of the chemokines CCL19 and CCL21 (Jang et al., 2006, Johansson-Lindbom et al., 2005). Murine CD103⁺ DCs (both CD11b⁺ and CD11b⁻ CD8α⁺ subsets) and CD103⁻ DCs from large intestinal LP upregulate CCR7 expression following stimulation with LPS (Rivollier et al., 2012). Furthermore, both CD103⁺ and CD103⁻ DC subsets are identified in the afferent lymphatics draining the murine small intestine (Cerovic et al., 2012). Following oral inoculation of mice with *Salmonella typhimurium*, CD103⁺ DCs are the first cells present within the MLN to contain detectable bacteria, and the arrival of these cells is significantly reduced in *Ccr7^{-/-}* mice (Bogunovic et al., 2009, Johansson-Lindbom et al.,

2005). This is consistent with the ability of murine CD103⁺ DCs to collect *Salmonella typhimurium* directly from the epithelium and then migrate to the draining MLN (Farache et al., 2013).

Migration of murine intestinal CD103⁺ DCs occurs constitutively at low levels in the steady state, and is enhanced by microbial stimulation (Schulz et al., 2009). This therefore allows a continuous surveillance of intestinal antigen in the steady state that may be augmented during a pathogenic challenge. Maturation may take place as DCs are en route to the MLNs as murine MLN DCs display elevated expression of CD40, CD80 and CD86 compared with their LP counterparts (Jang et al., 2006), whilst CD103⁺ DCs in murine MLNs also display a more mature phenotype than SI-LP CD103⁺ DCs (Jaensson et al., 2008). Furthermore, CD103⁺ DCs collected from murine cannulated afferent lymphatics express intermediate levels of co-stimulatory molecules (Cerovic et al., 2012).

Populations of DCs are present within GALT including PPs in mice. This includes a dense network of DCs in the subepithelial dome (SED) region immediately below the FAE (Kelsall and Strober, 1996). A subset of DCs within the SED expresses lysozyme, which damages bacterial peptidoglycan, and this cell population extends cellular protrusions through transcellular pores in M cells to internalize apical bacterial and particulate antigen (Lelouard et al., 2010, Lelouard et al., 2012). This population is CD11c⁺ CD11b⁺ F4/80⁻ CD8 α ⁻ and expresses CX₃CR1, consistent with a CD103⁻ DC phenotype (although CD103 expression is not defined in these studies). Lysozyme expressing DCs also capture apoptotic epithelial cells including M cells (Lelouard et al., 2010).

DCs are also found in the T cell-rich interfollicular regions (IFRs) of murine PPs, which may include mature DCs originating from the SED or a distinct DC subset (Kelsall and Strober, 1996). Following intragastric administration of the commensal bacteria *E. cloacae*, bacteria are detected within both CD8 α^+ and CD8 α^- DC populations of the PP but not from SI-LP (Macpherson and Uhr, 2004), indicating that PPs are the primary route of sampling of commensal bacteria. Furthermore, these bacteria may be cultured *in vitro* following purification of DCs and so are retained alive within DCs following capture (Macpherson and Uhr, 2004). Following uptake of bacteria, PP DCs display CCR7-dependent migration to the draining MLNs and are retained there (see Figure 1.3). This prevents the dissemination of live bacteria to the rest of the body (Macpherson and Uhr, 2004).

Murine PP DCs may interact directly with B cells and induce T cell-independent IgA production, or else enlist the help of T cells to drive germinal centre formation and T cell-dependent IgA production (Macpherson et al., 2000, Macpherson and Uhr, 2004). This is discussed further in section 1:8.6.

As discussed in section 1:5.2, murine intestinal CX₃CR1-GFP^{hi} M Φ do not migrate in draining lymphatics in the steady state (Schulz et al., 2009). However, mice pre-treated with antibiotics followed by oral administration of invasion-deficient *Salmonella typhimurium* display significantly increased levels of bacteria in MLNs, and the migration of CX₃CR1-GFP^{hi} M Φ is implicated in this process (Diehl et al., 2013). The intestinal microbiota may therefore suppress the migration of CX₃CR1-GFP^{hi} M Φ in the steady state, though further studies are required to rule out a role for migratory CX₃CR1-GFP^{int} CD103⁻ DCs.

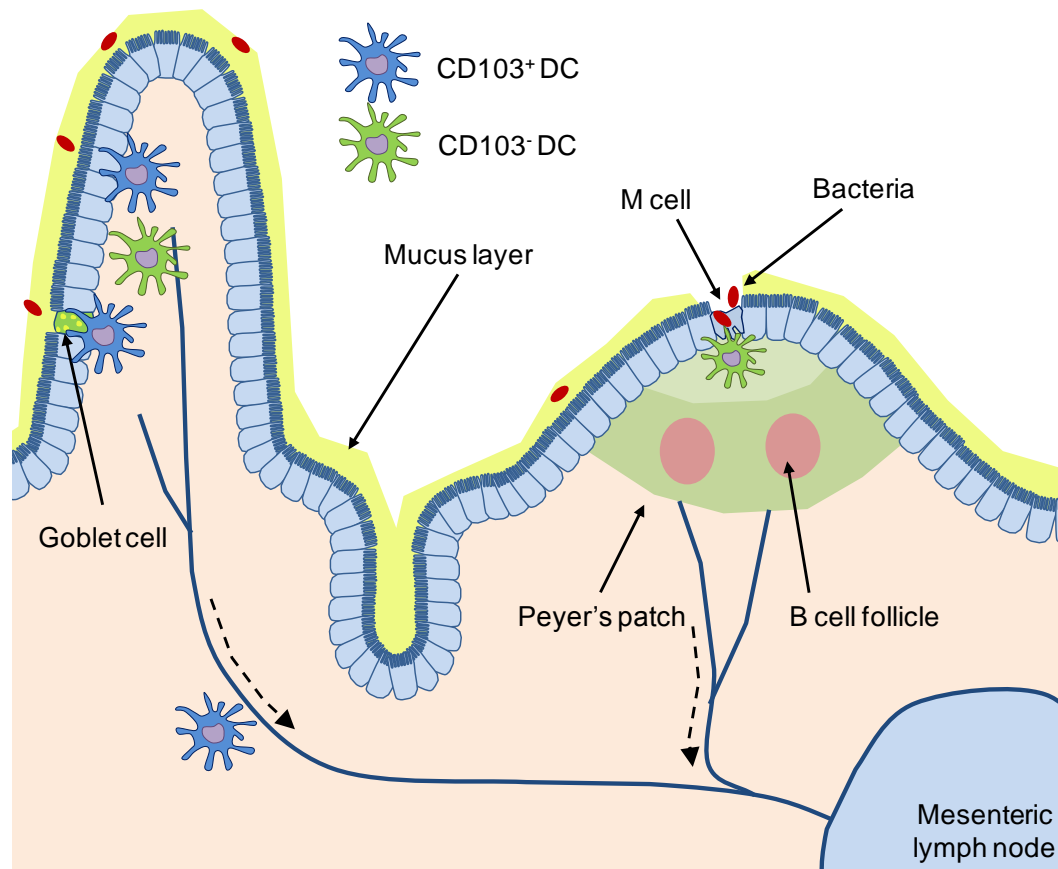


Figure 1.3: Antigen sampling by murine small intestinal DCs. CD103⁺ DCs interact with epithelial cells in response to luminal *Salmonella*, and collect particulate antigen from goblet cell-associated antigen passages (GAPs). CD103⁻ DCs extend dendrites through M cells in Peyer's patches to sample luminal bacteria. Following antigen sampling, both CD103⁺ and CD103⁻ DCs migrate in draining lymphatics to the MLNs. Adapted from Mowat, 2003.

1:6.3 Human intestinal dendritic cells

Conventional DCs in the human intestinal mucosa are identified as HLA-DR⁺ (MHC class II⁺), CD11c⁺ and by a lack of markers associated with other cell lineages (CD3 – T cells, CD14 – monocytes/MΦ, CD19 – B cells, CD34 – haematopoietic and endothelial cells, CD56 – NK cells). Following extraction from large intestinal mucosa, DCs

undergo maturation during overnight culture, up-regulating expression of CD40, CD80, CD83, CD86 and HLA-DR (Bell et al., 2001).

Following removal of epithelial cells from human intestinal biopsies by EDTA treatment, a range of cells including lymphocytes and highly activated DCs exit the tissue and may be collected for analysis (Bell et al., 2001). At least some of this cell migration occurs through pores in the basement membrane (Mahida et al., 1997). It is possible that this DC migration is a parallel of the ability of murine intestinal CD103⁺ DCs to patrol the epithelium in response to the presence of lumenal *Salmonella* (Farache et al., 2013), although this activity has yet to be demonstrated in humans.

DCs within the human large intestinal mucosa express significantly lower levels of TLR2 and TLR4 compared with blood DCs (Hart et al., 2005). This indicates that expression of these TLRs is down-modulated following entry of DCs into the intestinal mucosa.

A subset of myeloid DCs expressing blood dendritic cell antigen (BDCA-1; CD1c) has been observed in the human large and small intestine (Dillon et al., 2010). These are a subset of Lin⁻ HLA-DR⁺ CD11c⁺ cells and are predominantly CD103⁻. Furthermore, following overnight culture they upregulate the co-stimulatory molecules CD40 and CD86, the marker of mature human DCs CD83 as well as CCR7, suggesting they represent the human equivalent of murine CD103⁻ DCs. CD1c⁺ DCs are unresponsive to TLR4 or TLR5 stimulation, but produce TNF α and IL-6 following stimulation with the TLR2 ligand bacterial lipoprotein or the TLR7/8 ligand single stranded (ss)RNA (Heil et al., 2004, Takeuchi et al., 1999, Dillon et al., 2010). The TLR7/8 agonist also induces IL-23 production by CD1c⁺ DCs, whereas the TLR2 agonist induces IL-10 production (Dillon et al., 2010). This indicates that whilst myeloid DCs within the human intestinal

mucosa tolerate low levels of bacterial products such as LPS, the presence of particular ligands including ssRNA which suggest the presence of a viral infection triggers rapid cytokine production which then shapes the immunological response.

CD103⁺ DCs have been identified in human MLNs, and display increased expression of the maturation-associated markers CD40 and CD83 compared with their MLN CD103⁻ DC counterparts (Jaensson et al., 2008). In addition, an increased proportion of human MLN CD103⁺ DCs are CCR7⁺ compared with CD103⁻ DCs (Iliev et al., 2009). This is consistent with CD103⁺ DCs having migrated from the intestinal mucosa, with MLN CD103⁻ DCs as potentially blood-derived.

As in the mouse, a population of DCs expressing lysozyme is present in human PPs (Lelouard et al., 2010) and may therefore contribute to intestinal homeostasis through direct sampling of luminal bacteria.

A subset of myeloid DCs present in human blood defined by expression of CD141 (BDCA-3) is efficient at cross presentation of soluble antigen as well as antigen derived from virus-infected necrotic cells to CD8⁺ T cells. It is therefore proposed as the human equivalent of murine CD103⁺ CD11b⁻ CD8α⁺ DCs (Bachem et al., 2010, Jongbloed et al., 2010). Murine CD103⁺ CD11b⁻ CD8α⁺ DCs have also been shown to express Clec9A, a C-type lectin that binds necrotic cells via interaction with filamentous actin (Zhang et al., 2012). CLEC9A⁺ DCs are present in human PP, as well as in the SI-LP, suggesting a degree of species overlap (Poulin et al., 2012).

1:7 Ontogeny of intestinal mononuclear phagocytes

1:7.1 Ontogeny of murine intestinal mononuclear phagocytes

Murine intestinal DCs and M Φ populations originate from haematopoietic stem cells (HSCs) in the bone marrow following a complex process of differentiation. In mice, HSCs are self renewing and long-lived, generating short lived HSCs and then multipotent progenitor (MPP) cells. At this point, differentiation of these cells splits into the common lymphoid progenitor (CLP) which generates all lymphoid cells and the common myeloid progenitor (CMP). The CMP may differentiate into the megakaryocyte and erythrocyte progenitor (MEP) which ultimately generates erythrocytes and platelets, or alternatively the CMP becomes the granulocyte and macrophage progenitor (GMP) and subsequently all myeloid cell populations (reviewed by King and Goodell, 2011). GMPs may differentiate into the macrophage DC precursor (MDP) which in turn may then become a Ly6C^{hi} or Ly6C^{lo} monocyte, or alternatively the common DC precursor (CDP) (reviewed by Geissmann et al., 2010). The CDP may then differentiate into either a plasmacytoid DC (pDC) or a conventional DC precursor (pre-cDC), but will not become a monocyte (Liu et al., 2009, Naik et al., 2007, Onai et al., 2007). Intestinal CD103⁺ and CD103⁻ DCs are classed as conventional DCs (cDCs), distinguishing this group from pDCs which produce large quantities of type I interferon (IFN) upon interaction with viral products as part of a potent anti-viral response but possess a significantly lower ability to activate T cells as compared with cDCs (reviewed by Colonna et al., 2004). These differentiation pathways are summarized in Figure 1.4.

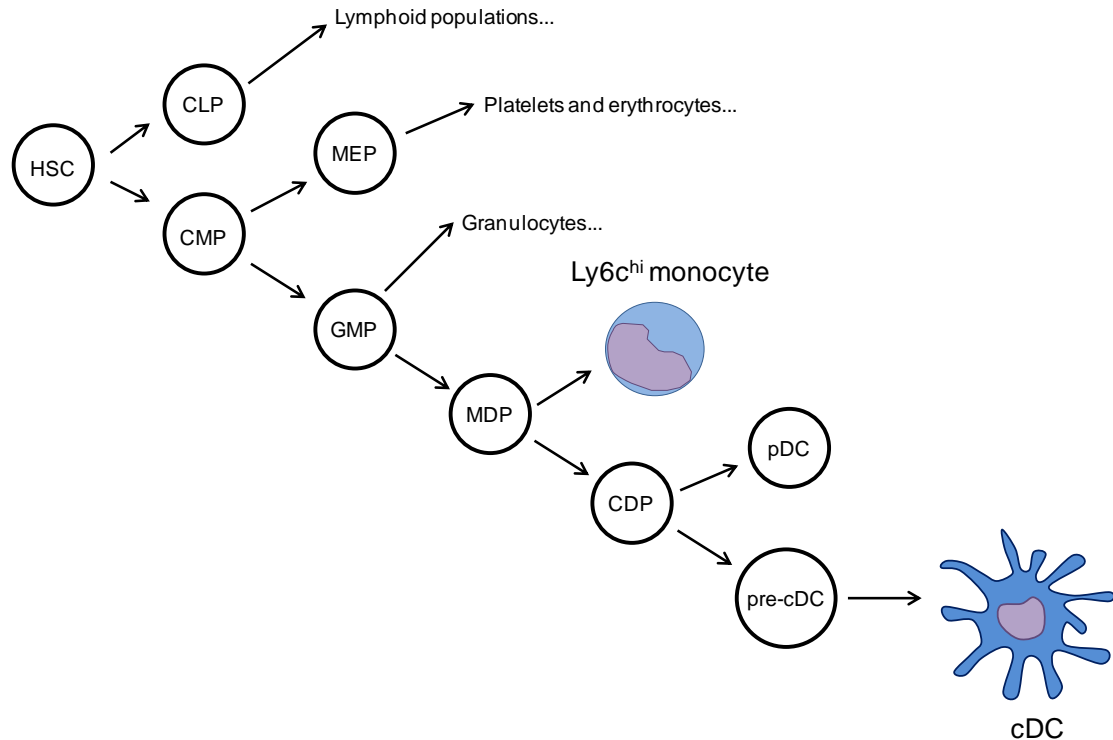


Figure 1.4: Haematopoiesis of murine Ly6C^{hi} monocytes and DCs in the murine bone marrow. Haematopoietic stem cells (HSCs) generate the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CMP generates megakaryocyte and erythrocyte progenitor (MEP) which generates platelets and erythrocytes, the CMP also generates the granulocyte macrophage progenitor (GMP), which in turn generates all granulocyte populations and the macrophage DC progenitor (MDP). MDP generates Ly6C^{hi} monocytes, which exit the bone marrow under the control of CCR2 and enter the intestinal mucosa in the steady state to give CX₃CR1^{hi} MΦ. MDP also gives the common DC progenitor (CDP) which generates either plasmacytoid DCs (pDC) or the pre-conventional DC (pre-cDC) which enters blood circulation and ultimately generates conventional DCs (cDC) such as CD103⁺ DCs within peripheral tissues including the intestinal mucosa.

The pre-cDC exits the bone marrow and enters blood circulation (Liu et al., 2009, Naik et al., 2006). Injection of MDPs, CDPs or pre-cDCs into mice generates both CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ CD8α⁺ SI-LP DCs (Bogunovic et al., 2009). Differentiation of MDPs to intestinal CD103⁺ DCs is particularly apparent in mice in which CD11c⁺ cells (including DCs and some MΦ populations) are first depleted (Bogunovic et al., 2009). This requires transgenic mice in which the diphtheria toxin receptor (DTR) is

expressed by CD11c⁺ cells, such that administration of diphtheria toxin selectively ablates CD11c⁺ cells (Jung et al., 2002). This may promote differentiation of CD103⁺ DCs through the creation of a 'niche' which these cells may then fill, however it is also possible that this reflects a non-physiological response.

Murine SI-LP CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ CD8α⁺ DCs increase in number in response to fms-like tyrosine kinase receptor-3 ligand (Flt3L) administration (Bogunovic et al., 2009, Cerovic et al., 2012). This is consistent with increased numbers of pre-cDCs in mice following Flt3L administration (Liu et al., 2009). GM-CSF also increases the numbers of murine SI-LP CD103⁺ DCs (Schulz et al., 2009). Intestinal CD103⁺ DC subsets do not however require M-CSF for their development (Bogunovic et al., 2009).

Due to the difficulty of distinguishing conventional murine intestinal CD103⁻ DCs from CD103⁻ CD11c⁺ MΦ in earlier studies in which DCs were defined as CD11c⁺ MHC II⁺ cells, less is known about the development of CD103⁻ DCs. However, Flt3L administration increases the numbers of murine SI-LP CD103⁻ DCs (Bain et al., 2013, Cerovic et al., 2012), indicating that they share a developmental pathway with CD103⁺ DCs.

A small proportion of intestinal CD103⁺ DCs may be derived from Ly6C^{hi} monocytes in CD11c-depleted mice in the steady state (Bogunovic et al., 2009, Rivollier et al., 2012). This may indicate further complexity in the differentiation pathway of these cells. In contrast, a large proportion of adoptively transferred Ly6C^{hi} monocytes differentiate into intestinal CX₃CR1-GFP^{hi} MΦ in mice in the steady state, although again the demonstration of this requires either prior depletion of CD11c⁺ cells or the use of CCR2^{-/-} mice (Rivollier et al., 2012, Bain et al., 2013, Tamoutounour et al., 2012, Zigmond et al., 2012). Differentiation of Ly6C^{hi} monocytes into CX₃CR1-GFP^{hi} MΦ within the

intestinal mucosa occurs in conjunction with upregulation of MHC II, F4/80, CX₃CR1 and CD64, with variable amounts of CD11c and concomitant loss of Ly6C (Rivollier et al., 2012, Bain et al., 2013, Tamoutounour et al., 2012, Zigmond et al., 2012). In contrast with DCs, murine intestinal CX₃CR1^{hi} MΦ do not expand in response to or require Flt3L or GM-CSF for differentiation in mice, but are dependent on M-CSF (Schulz et al., 2009, Bogunovic et al., 2009).

Expression of the transcription factor zDC (*Zbtb46*, *Btbd4*) was recently identified in murine pre-cDCs and cDCs but not MDPs or monocytes (Meredith et al., 2012a). Depletion of zDC⁺ cells using a zDC-DTR approach reduced numbers of CD103⁺ CD11b⁻ DCs in the SI-LP to approximately 6 % of their level in wild-type mice. In contrast, CD103⁺ CD11b⁺ DCs were reduced to 38 % of their level in wild-type mice (Meredith et al., 2012a). This indicates that majority of murine intestinal CD103⁺ CD11b⁻ DCs maintain significant levels of *Zbtb46* expression, whereas expression of *Zbtb46* is more variable within the murine intestinal CD103⁺ CD11b⁺ DC subset. However, the proportion of both DC subsets is not significantly different in *Zbtb46*^{-/-} mice, indicating that zDC may be involved in DC function as opposed to being required for their differentiation (Meredith et al., 2012a). Indeed, zDC expression is associated with suppression of MHC class II expression in the immature DC state (Meredith et al., 2012b).

Murine SI-LP CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ DCs are further distinguished by a range of transcription factors. For example, CD103⁺ CD11b⁻ DCs express significantly greater levels DNA-binding protein inhibitor 2 (*Id2*), *IRF8*, and *Batf3* compared with CD103⁺ CD11b⁺ DCs (reviewed by Persson et al., 2010). In contrast, murine SI-LP CD103⁺ CD11b⁺ DCs express elevated levels of *IRF4* compared with CD103⁺ CD11b⁻ DCs, and *IRF4* expression appears to promote survival of CD103⁺ CD11b⁺ DCs

(Persson et al., 2013). IRF4 expression is also observed in myeloid DCs within the human intestinal mucosa, suggesting a transcriptional overlap between mouse and man within these cells (Schlitzer et al., 2013, Persson et al., 2013).

Recent evidence indicates that RA may regulate the generation of a subset of pre-cDCs within the murine bone marrow expressing $\alpha_4\beta_7$ and which are predisposed to differentiate into intestinal CD103⁺ DCs (Zeng et al., 2012). Moreover, the differentiation of pre-cDCs into CD103⁺ CD11b⁺ DCs and CD103⁺ CD11b⁻ CD8 α ⁺ DCs in the murine SI-LP is also regulated by the presence of RA, with RA favouring the CD103⁺ CD11b⁺ DC phenotype (Klebanoff et al., 2013).

1:7.2 Ontogeny of human intestinal mononuclear phagocytes

Human intestinal resident M Φ are likely to derive from circulating CD14⁺ monocytes, which are the equivalent of murine Ly6C^{hi} monocytes (Ziegler-Heitbrock et al., 2010). Indirect evidence of differentiation derives from *in vitro* experiments in which human CD14⁺ monocytes cultured in intestinal stromal cell-conditioned medium develop an intestinal resident M Φ -like phenotype associated with loss of expression of CD14, low or non-responsiveness to microbial stimulation combined with strong phagocytic and bactericidal activity (Smythies et al., 2005). This differentiation process requires TGF- β , which is produced in by intestinal epithelial cells and mast cells (Smythies et al., 2005). Moreover, TREM-1 expression by human CD14⁺ monocytes is down-regulated in culture with TGF- β in the presence of IL-10 (Schenk et al., 2005). TGF- β signalling leads to phosphorylation of Smad2 and Smad3 proteins, which recruits Smad4 and the whole complex then translocates into the nucleus where it influences gene expression (reviewed by Massague, 1998). This process is inhibited by the presence of Smad7 in the cytosol, which inhibits Smad2/3 phosphorylation (reviewed by Massague, 1998).

Human intestinal resident M Φ display reduced levels of cytosolic Smad7 compared with blood monocytes, indicating that they experience constitutive TGF- β signalling within the intestinal mucosa (Smythies et al., 2010). Overall, this indicates that human CD14⁺ monocytes arriving in the intestinal mucosa from circulation undergo a differentiation process mediated by TGF- β that generates a resident M Φ phenotype specialised for the intestinal mucosa.

This system therefore appears to be similar to the mouse, where circulating Ly6C^{hi} monocytes enter the intestinal mucosa and differentiate into intestinal resident CX₃CR1-GFP^{hi} M Φ in *Cx3cr1*^{+/GFP} mice in the steady state (Bain et al., 2013, Tamoutounour et al., 2012, Rivollier et al., 2012).

1:8 Lymphocyte activation by intestinal DCs

Intestinal DCs interact with populations of naive T cells within draining MLN and present antigen obtained from within the intestinal mucosa in the context of MHC class I and MHC class II molecules. This process leads to expansion of T cells expressing TCRs specific for antigen derived from the intestinal mucosa. In addition, the subsequent activity of the responding T cells is also determined by cytokines produced by intestinal DCs during this activation event. This section will give an overview of the T cell subsets induced within GALT and MLNs by intestinal DCs (summarised in Figure 1.5) as well as discussing the role of these cells within the intestinal mucosa.

1:8.1 T_H1 cells

T_H1 cells play an important role in the eradication of intracellular pathogens such as *Listeria monocytogenes* and *Leishmania major*, and are characterised by expression of

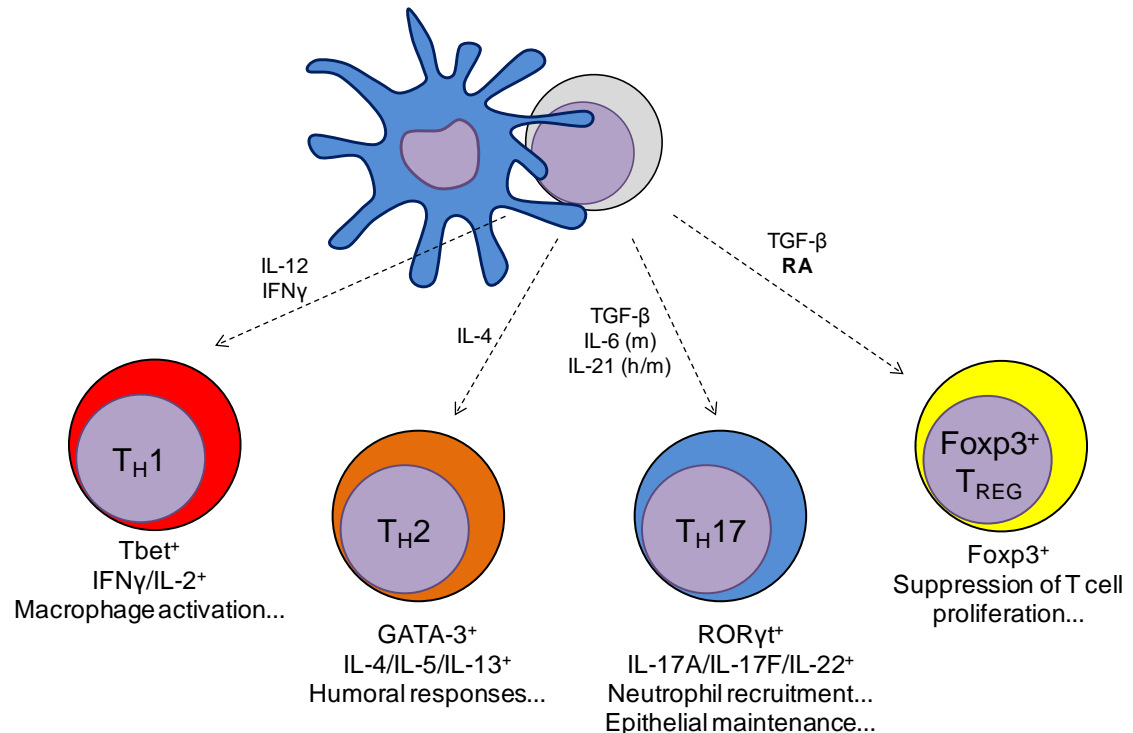


Figure 1:5: Induction of CD4⁺ T cell lineages by dendritic cells. Activation of naive CD4⁺ T cells in the presence of IL-12 and IFN γ induces T_H1 cells, which produce IFN γ , TNF α and IL-2 under the control of Tbet and mediate macrophage activation for defence against intracellular bacterial pathogens. IL-4 induces T_H2 cells which produce IL-4, IL-5 and IL-13 under the control of GATA-3, and support humoral responses and IgE class switching required for defence against extracellular pathogens e.g. helminths. TGF- β and IL-6 or IL-21 in mice (TGF- β and IL-21 in humans) induces T_H17 cells which produce IL-17A, IL-17F and IL-22 under the control of ROR γ t, and mediate defence against extracellular bacteria and fungal pathogens through neutrophil recruitment and increased neutrophil production in the bone marrow. RA enhances the TGF- β -mediated induction of Foxp3⁺ T_{REG} in mice which mediate suppression of effector T cell responses for the maintenance of homeostasis and the induction of oral tolerance.

the cytokines IFN γ , TNF α and IL-2 (reviewed by Szabo et al., 2003). One essential role of IFN γ is to activate macrophages, resulting in increased phagocytosis, increased MHC class I and II expression, and induction of IL-12 and nitric oxide, and superoxide which are all important in the eradication of intracellular pathogens (reviewed by Boehm et al., 1997).

Naive CD4⁺ T cells are induced into the T_H1 phenotype in the presence of IL-12, which signals through the IL-12 receptor composed of IL-12Rβ1 and IL-12Rβ2 chains (Chua et al., 1994, Presky et al., 1996). Naive CD4⁺ T cells upregulate the IL-12 receptor upon stimulation through the TCR, which renders them sensitive to IL-12 signalling (Presky et al., 1996). IFNγ plays an important role in supporting induction of the T_H1 phenotype through induction of the transcription factor T box expressed in T cells (Tbet) (Lighvani et al., 2001, Afkarian et al., 2002). Tbet induces expression of IL-12Rβ2 and so promotes IL-12 signalling in naive CD4⁺ T cells (Mullen et al., 2001, Afkarian et al., 2002). IL-12 then supports the T_H1 phenotype through promoting cell survival, division prolonging the production of IFNγ (Mullen et al., 2001). In addition, IL-12 signalling induces expression of the IL-18 receptor on T_H1 cells (Yoshimoto et al., 1998). IL-18 synergizes with IL-12 to promote IFNγ production by T_H1 cells (Robinson et al., 1997).

Murine CD103⁺ CD11b⁺ DCs collected from afferent lymphatics draining the SI-LP are poor inducers of IFNγ production in CD4⁺ T cells *in vitro* (Cerovic et al., 2012). However, stimulation of SI-LP CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ CD8α⁺ DCs with CpG ODN leads to significant induction of IFNγ by CD4⁺ T cells (Fujimoto et al., 2011). In contrast, CD103⁺ DCs collected from the afferent lymphatics draining the SI of rats efficiently induce IFNγ production by naive CD4⁺ T cells without prior stimulation (Milling et al., 2009). This may indicate that CD103⁺ DCs in rats are more pro-inflammatory in the steady state than in mice, or alternatively that they are more sensitive to cellular stress associated with the surgical procedure.

CD103⁻ DCs both from the murine large intestinal LP and in lymphatics draining the SI-LP induce strong IFNγ production from CD4⁺ T cells *in vitro*, without the need for prior stimulation from TLR ligands (Rivollier et al., 2012, Cerovic et al., 2012). When stimulated with anti-CD40 *in vitro* to replicate a T cell interaction, murine CD103⁻ DCs

from the afferent lymphatics draining the SI-LP express increased levels of L-12p35, IL-12/23p40 and IL-23p19 required for expression of both IL-12 and IL-23, compared with both CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ CD8 α ⁺ DCs collected from the same afferent lymphatics (Cerovic et al., 2012).

Murine MLN CD103⁻ DCs induce significantly greater levels of IFN γ production by naive CD4⁺ T cells compared with MLN CD103⁺ DCs (Laffont et al., 2010). This is also observed with human MLN CD103⁻ and CD103⁺ DCs (Iliev et al., 2009), indicating that in the steady state CD103⁺ DCs have a less inflammatory phenotype than CD103⁻ DCs in both mice and humans.

1:8.2 T_H2 cells

T_H2 cells are characterized by expression of a range of cytokines including IL-4, IL-5, IL-9, IL-13 and IL-25 (IL-17E) (reviewed by Paul and Zhu, 2010). They are primarily involved in defence against parasites through induction of IL-4 dependent B cell class switching to IgE. IgE immune complexes cross link high affinity Fc ϵ RI on innate cells including mast cells and basophils, leading to degranulation and the release of a wide range of cytokines, chemokines, histamine, serotonin and proteases involved in parasite clearance (reviewed by Paul and Zhu, 2010). Within the intestinal mucosa, T_H2 cells also recruit mast cells and eosinophils through production of IL-9 and IL-5, respectively (reviewed by Paul and Zhu, 2010).

T_H2 cells are induced from naive CD4⁺ T cells during T cell activation in the presence of IL-4, which signals through the transcription factor STAT6 and upregulates GATA-binding protein 3 (GATA-3), the master transcription factor for the T_H2 lineage (Zheng and Flavell, 1997, Zhang et al., 1997). IL-2 signalling through STAT5 also plays an

important role in this process (Zhu et al., 2003, Cote-Sierra et al., 2004), although phosphorylation of STAT5 by IL-7 or thymic stromal lymphopoietin (TSLP) can replace IL-2 in mice (reviewed by Rochman et al., 2009).

The extent to which DCs regulate IL-4-mediated induction of T_H2 cells from naive CD4⁺ T cells remains controversial. Basophils in mice may act as antigen presenting cells and present antigen derived from IgE complexes to naive CD4⁺ T cells and promote the induction of T_H2 cells (Yoshimoto et al., 2009). Furthermore, basophils migrate to skin-draining lymph nodes in mice following stimulation with the cysteine protease papain and produce IL-4 and TSLP (Sokol et al., 2008a). Basophils may also play a key role in inducing an effective T_H2 response in mice to clear infection with the intestinal helminth *Trichuris muris* (Perrigoue et al., 2009). However, depletion of CD11c⁺ cells (including DCs and some MΦ subsets) in mice significantly reduces the induction of an effective T_H2 response to the helminth *Schistosoma mansoni*, whilst depletion of basophils has no effect on T_H2 induction (Phythian-Adams et al., 2010). Furthermore, adoptive transfer of bone-marrow derived DCs pulsed with antigen derived from the intestinal nematode *Nippostrongylus brasiliensis* induces a T_H2 response in naive mice (Balic et al., 2004). Production of TSLP by intestinal epithelial cells may condition intestinal DCs to make reduced levels of IL-12p40, favouring the induction of T_H2 cells in *Trichuris muris* infection (Taylor et al., 2009). Intestinal DCs are therefore likely to play a key role in the induction of T_H2 responses to helminth infection, however the nature of this induction remains to be defined in a greater level of detail (reviewed by Allen and Maizels, 2011).

1:8.3 T_H17 cells

Activation of murine naive CD4⁺ T cells in the presence of TGF-β and IL-6 induces a

T_H17 helper T cell phenotype based on production the cytokines IL-17A and IL-17F (Bettelli et al., 2006, Veldhoen et al., 2006, Mangan et al., 2006). In addition, T_H17 cells may be differentiated from naive CD4⁺ T cells with TGF- β in the presence of IL-21 (Korn et al., 2007, Nurieva et al., 2007, Zhou et al., 2007). T_H17 differentiation is under the transcriptional control of ROR γ t (Ivanov et al., 2006), which synergizes with ROR α (Yang et al., 2008d). T_H17 cells cultured with only TGF- β and IL-6 develop a non-inflammatory IL-17A⁺ IL-10⁺ phenotype, whilst secondary stimulation with IL-23 promotes commitment to the T_H17 lineage, expansion in cell numbers and a loss of IL-10 production (McGeachy et al., 2007, McGeachy et al., 2009).

T_H17 cells display a reciprocal relationship with Foxp3⁺ T_{REG} (discussed in section 1:8.5). Induction of T_H17 cells is suppressed by IL-2, whilst induction and expansion of Foxp3⁺ T_{REG} is enhanced (Setoguchi et al., 2005, Davidson et al., 2007, Zheng et al., 2007). Furthermore, induction of T_H17 cells is suppressed by retinoic acid (RA) whilst that of Foxp3⁺ T_{REG} is enhanced (Mucida et al., 2007, Schambach et al., 2007, Elias et al., 2008). Consistent with a high ability to produce RA, CD103⁺ CD11b⁺ DCs collected from the afferent lymphatics draining the SI-LP of mice are poor inducers of T_H17 cells *in vitro* (Cerovic et al., 2012). However, prior stimulation of SI-LP CD103⁺ CD11b⁺ DCs with flagellin or CpG ODN significantly enhances induction of T_H17 cells through enhanced production of IL-23 (Fujimoto et al., 2011). Moreover, murine SI-LP CD103⁺ CD11b⁺ DCs display a significantly greater ability to induce T_H17 cells compared with CD103⁺ CD11b⁻ DCs (Denning et al., 2011). As discussed in section 1:7.1, murine SI-LP CD103⁺ CD11b⁺ DCs express IRF4 which mediates survival of these cells (Persson et al., 2013). Mice deficient in IRF4 specifically in CD11c⁺ cells are deficient for CD103⁺ CD11b⁺ DCs leading to significantly reduced levels of T_H17 cells within the intestinal

mucosa (Persson et al., 2013). This indicates that murine intestinal CD103⁺ DCs play a key role in inducing T_H17 cells *in vivo*.

Activation of human naive CD4⁺ T cells in the presence TGF- β and IL-6 does not however induce a T_H17 phenotype (Acosta-Rodriguez et al., 2007, van Beelen et al., 2007, Wilson et al., 2007), which instead requires TGF- β and IL-21 (Yang et al., 2008a). The human T_H17 phenotype is also under the transcriptional control of ROR γ t (Wilson et al., 2007). Furthermore, RA suppresses induction of human T_H17 cells from naive CD4⁺ T cells (Kamada et al., 2009).

T_H17 cells in both mice and humans produce IL-17A, IL-17F, IL-22 and GM-CSF (Annunziato et al., 2007, Wilson et al., 2007). IL-17A and IL-17F play important roles in defence against extracellular bacterial and fungal pathogens in mice through indirect recruitment of neutrophils via induction of neutrophil chemokines including CXCL8 (IL-8) by stromal cells as well as induction of the production of further neutrophils from the bone marrow by inducing release of G-CSF from stromal cells (reviewed by Witowski et al., 2004). IL-22 production promotes the production of antimicrobial peptides by epithelial cells and therefore may help to enhance epithelial barrier function during an infection (Zheng et al., 2008).

The induction of T_H17 cells within the intestinal mucosa may be influenced by particular members of the microbiota. Mice harbouring segmented filamentous bacteria (SFB) possess significantly increased levels of T_H17 cells within the intestinal mucosa compared to mice without it (Ivanov et al., 2009). This increased presence of T_H17 cells associates with an increased ability to cope with *Citrobacter rodentium* infection (Ivanov et al., 2009).

1:8.4 Cytotoxic T lymphocytes

DCs present peptides derived from extracellular antigen in the context of MHC class I to naive CD8⁺ T cells as part of a process termed 'cross presentation' (reviewed by Joffre et al., 2012). The presence of IL-12 during this activation induces the differentiation of naive CD8⁺ T cells into cytotoxic T lymphocytes (CTLs) which have the ability to induce apoptosis of virally infected or malignant cells expressing their cognate peptide-MHC class I target through the release of granzyme and perforin reviewed by Kaech and Cui, 2012). Induction of the transcription factors Tbet and eomesodermin (EOMES) induces expression of IFN γ , TNF α , granzyme B, perforin and the chemokine receptors CXCR3 and CXCR4 as part of the CTL differentiation process (Intlekofer et al., 2005, Joshi et al., 2007).

CD103⁺ CD11b⁻ DCs collected from the murine large intestinal LP display a superior ability to induce activation of CD8⁺ T cells as compared with CD103⁺ CD11b⁺ DCs (Rivollier et al., 2012). Furthermore, murine CD103⁺ CD11b⁻ CD8 α ⁺ DCs collected from intestinal afferent lymphatics are also superior to CD103⁺ CD11b⁺ DCs in this regard (Cerovic et al., 2012). However, one study has demonstrated that CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ CD8 α ⁺ DCs collected from SI-LP have equivalent ability to activate CD8⁺ T cells following stimulation with the TLR9 ligand CpG oligodeoxynucleotides ODN (Fujimoto et al., 2011). Whether stimulation overcomes differences in CD8⁺ T cell activation or if collecting the cells from different compartments influences this activity is unclear.

Murine CD103⁺ CD11b⁺ DCs collected from afferent lymphatics draining the SI-LP are poor inducers of IFN γ production in CD8⁺ T cells *in vitro*. However, prior stimulation of these CD103⁺ CD11b⁺ DCs with the TLR2 ligand bacterial lipoprotein significantly

increases their ability to induce IFN γ production by CD8 $^{+}$ T cells (Cerovic et al., 2012). In contrast, CD103 $^{-}$ DCs from both the murine large intestinal LP and in lymphatics draining the SI-LP induce strong IFN γ production from CD8 $^{+}$ T cells *in vitro* without the need for prior stimulation (Rivollier et al., 2012, Cerovic et al., 2012). Injection of OVA-pulsed and CpG ODN-stimulated SI-LP CD103 $^{+}$ CD11b $^{+}$ DCs into the murine peritoneum leads to efficient induction of OVA-specific IFN γ^{+} CD8 $^{+}$ T cells *in vivo* (Fujimoto et al., 2011).

Targeting ovalbumin (OVA) to murine CD103 $^{+}$ DCs by conjunction of the antigen to an anti-CD103 monoclonal antibody and then administration by IP injection leads to proliferation of OVA-specific CD4 $^{+}$ and CD8 $^{+}$ T cells with low cytokine production in MLNs. However co-administration of anti-CD40 monoclonal antibody leads to efficient induction of OVA-specific IFN γ^{+} CD8 $^{+}$ T cells which are cytotoxic for OVA peptide-presenting splenocytes (Semmrich et al., 2011).

1:8.5 Regulatory T cells

A significant proportion of effector T cells in the steady state human intestinal mucosa possess a T $_{H1}$ phenotype with the potential to produce IFN γ upon stimulation (Rovedatti et al., 2009). Furthermore, during an acute gastrointestinal infection T $_{H1}$ cells are generated with specificity for microbiota-derived antigen and persist in the intestinal mucosa of mice for an extended period of time (Hand et al., 2012). The activity of these effector T cell populations is likely to be restrained in order to maintain intestinal homeostasis and prevent immunopathology. Subsets of regulatory T cells (T $_{REG}$) play important roles in restraining the activity of these cells (reviewed by Barnes and Powrie, 2009).

A major subset of T_{REG} in mice are defined by expression of the transcription factor Forkhead winged helix P3 (Foxp3) (Hori et al., 2003) and high expression of the IL-2 receptor- α chain CD25 (reviewed by Barnes and Powrie, 2009). Foxp3⁺ T_{REG} mediate suppression of T cell proliferation and cytokine production through a range of mechanisms. Contact-dependent mechanisms of suppression include surface expression of cytotoxic T lymphocyte associated antigen 4 (CTLA-4) which interacts with and down-regulates the co-stimulatory molecules CD80 and CD86 on APCs (Takahashi et al., 2000, Read et al., 2000), whilst contact-independent mechanisms include production of IL-10 (reviewed by Barnes and Powrie, 2009).

CD25^{hi} Foxp3⁺ T_{REG} are divided into natural T_{REG} (nT_{REG}) which develop in the thymus and are believed to express TCRs with specificities for self antigen, and inducible T_{REG} (iT_{REG}) which develop in the periphery from naive CD4⁺ T cells and therefore allow expansion of repertoire of antigens which may be subject T_{REG} responses (reviewed by Barnes and Powrie, 2009). Naive CD4⁺ T cells are induced into Foxp3⁺ iT_{REG} during T cell activation in mice in the presence of TGF- β (Chen et al., 2003), which is further enhanced with RA (Coombes et al., 2007, Sun et al., 2007, Mucida et al., 2007). Murine CD103⁺ DCs from both the SI-LP and MLN possess a specialised ability to induce Foxp3⁺ iT_{REG} *in vitro* through expression of the integrin $\alpha_v\beta_8$ which activates latent TGF- β (Travis et al., 2007, Paidassi et al., 2011, Worthington et al., 2011). This is further supported by the provision of RA through enhanced expression of *Aldh1a2*, encoding RALDH2 (Coombes et al., 2007, Sun et al., 2007).

Murine CD103⁺ CD11b⁺ DCs but not CD103⁺ CD11b⁻ CD8 α ⁺ DCs from SI-LP induce Foxp3⁺ T_{REG} from naive CD4⁺ T cells in the presence of exogenous TGF- β (Fujimoto et al., 2011). This may reflect the low ability of murine SI-LP CD103⁺ CD11b⁻ CD8 α ⁺ DCs to produce RA (Fujimoto et al., 2011). Furthermore, murine large intestinal LP CD103⁺

CD11b⁺ DCs induce Foxp3⁺ T_{REG} from naive CD4⁺ T cells but in this study a further population of CD103⁺ CD11b⁻ CD8 α ⁻ DCs and CD103⁻ DCs were unable to do so (Rivollier et al., 2012). In comparison with CD103⁺ DCs, murine resident M Φ have a reduced ability to produce RA. They do however express *Aldh1a1* (encoding RALDH1), of which expression is greatest in the CD11c⁻ subset (Denning et al., 2011). Their ability to induce Foxp3⁺ T_{REG} is suppressed when RA signalling is inhibited, suggesting that this may contribute to a regulatory phenotype (Denning et al., 2007).

The combined action of TGF- β and RA exert complex epigenetic regulation of Foxp3 expression in murine CD4⁺ T cells. RA enhances TGF- β -mediated induction of murine Foxp3⁺ iT_{REG} acts through phosphorylation of ERK1/2, leading to increased histone methylation and acetylation at the *Foxp3* promoter (Lu et al., 2011). RA and TGF- β induce expression of a particular profile of micro RNA molecules (miRNA) which increase the stability of the Foxp3⁺ T_{REG} phenotype (Takahashi et al., 2012). It has also been proposed that RA does not act on iT_{REG} directly, but inhibits the production of cytokines such as IFN γ by neighbouring effector cells which normally suppress the induction of Foxp3 (Hill et al., 2008).

Murine SI-LP and MLN CD103⁺ DCs also express the enzyme indoleamine 2,3-dioxygenase (IDO), which is involved in tryptophan catabolism (Matteoli et al., 2010). This enzyme activity contributes to the induction of Foxp3⁺ iT_{REG} in mice *in vivo*, indicating that IDO acts to complement TGF- β and RA in the induction of these cells (Matteoli et al., 2010).

Murine Foxp3⁺ nT_{REG} and iT_{REG} are distinguished in the literature by expression of the transcription factor Helios, such that Foxp3⁺ nT_{REG} are Helios⁺ (Sugimoto et al., 2006, Thornton et al., 2010). However, subsequent reports indicate that Helios is transiently

upregulated during the activation and proliferation of murine CD4⁺ T cells, including Foxp3⁺ iT_{REG}, indicating that expression of Helios is insufficient to distinguish murine nT_{REG} and iT_{REG} (Akimova et al., 2011, Gottschalk et al., 2012). There is controversy as to whether the population of Foxp3⁺ T_{REG} in murine intestinal LP is predominantly of nT_{REG} or iT_{REG} origin. Germ-free mice contain equivalent proportions of MLN and colonic T_{REG} populations when compared with mice containing intestinal microbiota (Min et al., 2007, Round and Mazmanian, 2010). However, a subset of colonic Foxp3⁺ T_{REG} in microbiota-containing mice express TCRs specific for microbiota-derived antigen (Lathrop et al., 2011). Furthermore, these specific TCR sequences do not support nT_{REG} formation when expressed in *Rag*^{-/-} thymocytes, and adoptive transfer of CD4⁺ T cells expressing such TCRs into *Rag*^{-/-} mice recapitulates T-cell transfer colitis (see 1:14.5.2) (Lathrop et al., 2011). Overall, this indicates that at least some of the murine colonic Foxp3⁺ T_{REG} population is induced in the periphery, potentially under the influence of CD103⁺ DCs. It is therefore proposed that the murine intestinal mucosa comprises a T_{REG} ‘niche’, such that in absence of microbiota-derived antigen leading to induction of Foxp3⁺ iT_{REG}, it is instead filled with nT_{REG} (Lathrop et al., 2011).

However, this has recently been challenged by a report in which the TCR was sequenced from large numbers of Foxp3⁺ and Foxp3⁻ CD4⁺ T cells in the murine intestine and thymus. This indicates that the majority of Foxp3⁺ T_{REG} in the murine intestine express TCR sequences that are identified within Foxp3⁺ T_{REG} in the thymus, suggesting that they represent thymus-derived nT_{REG} (Cebula et al., 2013). Modulation of intestinal microbiota through antibiotic treatment significantly alters the frequency of specific Foxp3⁺ T_{REG} TCR clones within the murine intestine, indicating a close association between antigen and respective T_{REG} population. However, these TCRs are again present in the thymus (Cebula et al., 2013). This therefore challenges the notion

that nT_{REG} recognise only self-antigen, and suggests a crucial role for Foxp3⁺ nT_{REG} in maintaining tolerance to microbiota-derived antigen. Murine MLN CD103⁺ DCs support the maintenance of CD25^{hi} Foxp3⁺ T_{REG} in addition to inducing this phenotype from naive CD4⁺ T cells (Coombes et al., 2007), and so may provide the link between microbiota-derived antigen and the relative abundance of specific Foxp3⁺ nT_{REG} populations within the intestinal mucosa.

The induction of Foxp3⁺ iT_{REG} by murine MLN CD103⁺ DCs is implicated in oral tolerance, in which mice develop systemic peripheral tolerance to a fed antigen e.g. ovalbumin (OVA) (reviewed by Mayer and Shao, 2004). This is a multi-step process that requires sampling of OVA from the intestinal lumen and CCR7-dependent migration of CD103⁺ DCs into the MLNs (Worbs et al., 2006). Furthermore, the gut-homing of Foxp3⁺ iT_{REG} is then required as this does not develop in mice deficient in *Ccr9*^{-/-} or *Itb7*^{-/-} mice (Cassani et al., 2011, Hadis et al., 2011). Finally, the arrival of Foxp3⁺ iT_{REG} is followed by a process of expansion within the intestinal mucosa under the influence of IL-10 derived from resident CX₃CR1-GFP^{hi} MΦ (Hadis et al., 2011).

Administration of the human commensal bacterium *Bacteroides fragilis* into germ-free mice leads to the induction of IL-10-producing Foxp3⁺ T_{REG}. Furthermore, this conversion requires expression of polysaccharide A (PSA) by these bacteria (Round and Mazmanian, 2010). Deletion of IL-10 specifically in Foxp3⁺ T cells renders mice susceptible to spontaneous large intestinal inflammation, particularly in the caecum (Rubtsov et al., 2008). This indicates that production of IL-10 is an important mechanism by which Foxp3⁺ T_{REG} maintain intestinal homeostasis in the steady state in mice. This is supported by the large presence of Foxp3⁺ IL-10⁺ and Foxp3⁻ IL-10⁺ CD4⁺ T cells in the murine small and large intestinal mucosa in the steady state which are not apparent in other peripheral tissues (Maynard et al., 2007). Furthermore, mice lacking

IL-10 expression in Foxp3⁺ T cells do not display the systemic auto-immunity of Scurfy mice indicating that distinct mechanisms prevent the activation and expansion of self-reactive effector T cells outside the intestine (Rubtsov et al., 2008).

Type 1 regulatory T cells (T_R1) represents an alternative regulatory T cell population in mice and humans and are defined by low proliferative capacity, high expression of IL-10 and low expression of TGF- β , IFN γ and IL-2 upon activation (Groux et al., 1997). They are induced in the presence of high concentrations of IL-10 and efficiently suppress the proliferation of T cells in a contact-independent mechanism involving both IL-10 and TGF- β (Groux et al., 1997). Murine T_R1 cells do not express Foxp3, and are therefore distinct from both Foxp3⁺ nT_{REG} and iT_{REG} (Vieira et al., 2004). The relationship between these subsets is controversial, however human peripheral blood CD25⁺ T_{REG} induce an IL-10 producing T_R1-like phenotype in naive CD4⁺ T cells during suppression assays (Dieckmann et al., 2002), indicating that Foxp3⁺ T_{REG} may support the induction of further regulatory T cell phenotypes.

In the presence of exogenous TGF- β , murine MLN CD103⁺ DCs efficiently induce Foxp3⁺ iT_{REG} but fail to induce the IL-10⁺ T_R1 phenotype from naive CD4⁺ T cells (Maynard et al., 2009). Inhibition of RA signalling during this stimulation however leads to efficient induction of IL-10⁺ T_R1 cells, whilst suppressing Foxp3⁺ iT_{REG} induction (Maynard et al., 2009). This indicates that the production of RA by MLN CD103⁺ DCs exerts reciprocal regulation of Foxp3⁺ iT_{REG} and T_R1 induction.

The importance of FOXP3⁺ T_{REG} in humans is indicated by the fatal autoimmunity of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) which is caused by mutations in the *FOXP3* gene (Bennett et al., 2001, Wildin et al., 2001). However, the identification of T_{REG} in humans is more difficult

in humans than in mice. This is due to transient expression of FOXP3 by activated human CD4⁺ T cells (Gavin et al., 2006, Allan et al., 2007, Pillai et al., 2007, Merlo et al., 2008) which does not associate with suppressive activity. Furthermore, induction of FOXP3 in naive CD4⁺ T cells with exogenous TGF- β does not induce suppressive activity (Tran et al., 2007). Additional markers including a lack of the IL-7 receptor (CD127) have been proposed to identify FOXP3⁺ T_{REG} in human blood (Liu et al., 2006). This complexity means that identification of T_{REG} in humans requires careful demonstration of regulatory properties.

Studies have identified populations of T_{REG} within the steady state human intestinal mucosa on the basis of high level expression of CD25 which are able to suppress the proliferation of CD25⁻ cells *in vitro* (Makita et al., 2004). Furthermore myofibroblasts within human colonic LP support expansion of FOXP3⁺ T_{REG} and the induction of FOXP3⁺ T_{REG} from naive CD4⁺ T cells (Pinchuk et al., 2011). Stimulation of human blood T_{REG} with flagellin through TLR5 increases suppressive activity, suggesting that interactions with microbial products in the intestinal mucosa may enhance the ability of T_{REG} to maintain homeostasis in the steady state (Crellin et al., 2005).

Nevertheless, human MLN CD103⁺ DCs induce a significantly greater number of FOXP3⁺ T_{REG} compared with CD103⁻ DCs (Iliev et al., 2009), which is consistent with the properties of murine MLN CD103⁺ DCs described above.

1:8.6 B cells

GALT is an important site of induction of IgA production by B cells. Murine DCs carrying live bacteria induce bacteria-specific IgA without T cell help (Macpherson et al., 2000). This process may be recapitulated *in vitro* with murine PP DCs and requires the production

of RA as well as IL-5 or IL-6 by these DCs (Mora et al., 2006). Whilst specific for bacterial antigen, this IgA is of relatively low affinity as compared with that generated by B cells with the help of T cells in the GALT. In this case, activated T cells interact with or help B cells which have identified a component of the same antigen through the surface B cell receptor (BCR). Co-stimulation provided as a result of this interaction results in the formation of a germinal centre, wherein the B cell undergoes class switch recombination from IgM to IgA and also somatic hypermutation in which multiple clones of the original B cell are generated but with a range of mutations in the variable region of their antibody. Those mutants expressing an antibody of greater affinity are positively selected, resulting in the expansion of a clone generating a high affinity IgA (reviewed by Pabst, 2012).

Activated B cells producing IgA differentiate into plasma cells and exit the GALT through draining lymphatics and enter the circulation via the thoracic duct. Following selective homing of these cells back into the gut (discussed in more detail in section 1:9.3), they remain there for an extended period of time and generate large quantities of sIgA which is passed through the epithelium by pIgR-mediated transcytosis (reviewed by Pabst, 2012).

Murine SI-LP CD103⁺ CD11b⁺ DCs efficiently induce the differentiation of naive B cells into IgA⁺ plasma cells *in vitro* following stimulation with CpG (ODN) through T cell-independent class switching (Fujimoto et al., 2011). In contrast, SI-LP CD103⁺ CD11b⁻ CD8 α ⁺ DCs do not efficiently induce this differentiation. IgA class-switching may be induced in the presence of RA and cytokines including IL-5 or IL-6 (Mora et al., 2006). The ability of CD103⁺ CD11b⁺ DCs to induce IgA class-switching may therefore relate to a superior ability to generate RA through enhanced *Aldh1a2* (encoding RALDH2) expression (Fujimoto et al., 2011).

Intraperitoneal (IP) injection of murine SI-LP CD103⁺ CD11b⁺ DCs pulsed with the model antigen ovalbumin (OVA) and stimulated with CpG (ODN) into mice followed by immunization with OVA leads to the generation of OVA-specific serum IgG and also sIgA as detected in faeces. In contrast, repeating this experiment with SI-LP CD103⁺ CD11b⁻ CD8 α ⁺ DCs generates only serum IgG, consistent the reduced ability of CD103⁺ CD11b⁻ CD8 α ⁺ DCs to generate the RA required for IgA class-switching. However, murine CD103⁺ CD11b⁻ CD8 α ⁺ DCs and CD103⁺ CD11b⁺ DCs collected from lymphatics draining the murine small intestine possess equivalent levels of RALDH activity, and induce a similar level of CCR9 expression on CD8⁺ T cells (Cеровic et al., 2012). They may therefore generate a similar degree of IgA class-switching upon interaction with B cells in the draining MLN. The reason for this difference in RA-producing ability is unclear, but may relate to whether cells are collected directly from the mucosa or en route to the draining MLN.

1:9 Lymphocyte migration

1:9.1 Migration of naive lymphocytes into GALT and MLN

DCs present within GALT and also those entering the draining MLNs interact with local populations of naive T cells. However, these lymphocyte populations are not static but actively circulate between lymphoid tissue and the blood stream in order to maximise the probability of an interaction with an antigen presenting cell presenting their cognate peptide-MHC ligand (reviewed by Agace, 2006).

The mechanism of entry of naive T cells into murine PP has been elucidated in detail, and follows the 'multi-step adhesion cascade model' (reviewed by Butcher and Picker, 1996). Naive T cells express CD62L (L-selectin), a glycoprotein of the C-type lectin

group which bind to specific carbohydrate moieties on selectin ligands. CD62L interacts with oligosaccharides containing sialyl-Lewis^x residues expressed on high endothelial venules (HEV) in lymph nodes (reviewed by (Butcher and Picker, 1996). Furthermore, CD62L interacts with sulphated glycans present on mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (Berg et al., 1993), an immunoglobulin gene superfamily member which is expressed constitutively by endothelial cells throughout the gastrointestinal tract of mice and humans including associated lymphoid tissue and MLNs (Briskin et al., 1997). In addition, naive T cells express low to intermediate levels of the integrin $\alpha_4\beta_7$ which is the principal receptor mediating interaction with MAdCAM-1 (Berlin et al., 1993). Low affinity interactions between L-selectin and MAdCAM-1 with support from $\alpha_4\beta_7$ -MAdCAM-1 interactions facilitate tethering and rolling of circulating naive T cells along the PP endothelial surface (Bargatze et al., 1995). Naive T cells are then exposed to additional factors including the chemokine CCL21 which is present on HEV within T cell zones of PPs and MLNs, and which signals through the chemokine receptor CCR7 expressed by naive T cells. CCL21-CCR7 signalling triggers a conformational change or activation of the integrin $\alpha_L\beta_2$ on naive T cells, enhancing the ability of $\alpha_L\beta_2$ to interact with intercellular adhesion molecule 1 (ICAM-1) (reviewed by Sigmundsdottir and Butcher, 2008). The result of these interactions is the complete arrest of naive T cells in the PP and MLN HEVs, followed by a process of transendothelial migration (diapedesis) which is not fully understood. This process is summarized in Figure 1.6.

1:9.2 Migration of effector T cells into intestinal mucosa

Following activation in GALT and MLNs, effector T cells display a significant shift in their migratory capacity. This includes downregulation of CD62L and CCR7, consistent

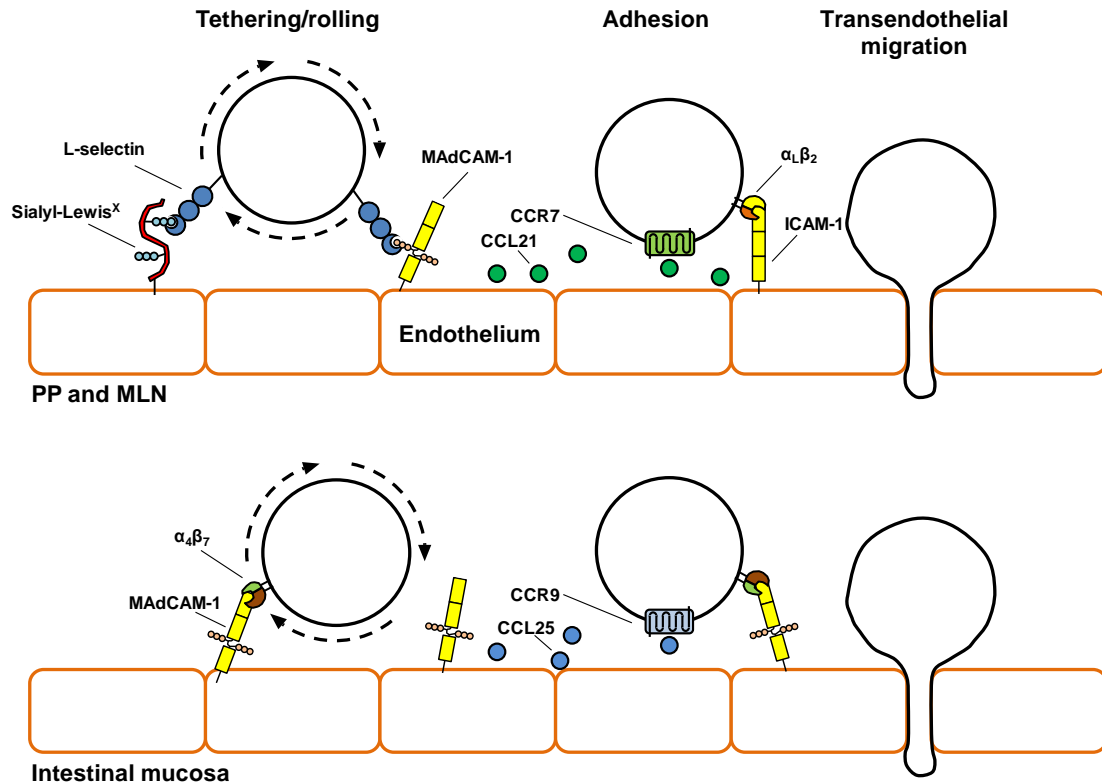


Figure 1.6: Migration of naive T cells and effector T cells into intestinal mucosa. *Upper panel:* Naive T cells express L-selectin which interacts with sialyl-Lewis^x residues expressed on high endothelial venules (HEVs) in PPs and MLNs, as well as with glycans of MAdCAM-1, leading to tethering and rolling along the endothelium. Low expression of $\alpha_4\beta_7$ by naive T cells contributes to tethering through interaction with MAdCAM-1 (not shown). Signalling by CCL21 present on HEVs through CCR7 on naive T cells induces activation of the $\alpha_L\beta_2$ integrin of naive T cells and increases affinity of this integrin for HEV-associated ICAM-1, bringing the cell to a stop. Transendothelial migration then transports the naive T cell to the interior of the tissue in a process which remains poorly understood. *Lower panel:* Naive T cells activated in GALT express high levels of $\alpha_4\beta_7$ which is sufficient to interact with MAdCAM-1 on vascular endothelial cells in the intestinal mucosa and induce tethering and rolling behaviour. Signalling of CCL25 through CCR9 promotes recruitment of lymphocytes to the small intestinal mucosa, and may act through modulation of $\alpha_4\beta_7$ -MAdCAM-1 interactions.

which a diminished ability to enter lymphoid tissue (reviewed by Masopust and Schenkel, 2013). Simultaneously, they are induced to express a gut-homing phenotype that allows them to home specifically into the intestinal mucosa once they enter blood

circulation via the thoracic duct (reviewed by Agace, 2006). This selective migration is a key part of the compartmentalisation of intestinal immune responses, where antigen sampled in a gut-specific context requires a gut-specific response. A subset of activated cells retain expression of both CD62L and CCR7, and so are able to continue to circulate through secondary lymphoid organs as central memory cells (T_{CM}) (reviewed by Masopust and Schenkel, 2013).

The first component of the T cell gut-homing profile is high level surface expression of the integrin $\alpha_4\beta_7$, facilitating expression of MAdCAM-1 present on endothelial cells throughout the gastrointestinal tract (Briskin et al., 1997). In murine PP HEVs, high level expression of $\alpha_4\beta_7$ is sufficient to induce rapid arrest of T cells through interaction with MAdCAM-1 (Bargatze et al., 1995). Mice treated with anti-MAdCAM-1 monoclonal antibodies have suppressed recruitment of adoptively transferred T cells into SI-LP and PPs (Haddad et al., 2003, Cassani et al., 2011).

The second component of the lymphocyte gut-homing profile is the chemokine receptor CCR9, a 7 transmembrane spanning G protein-coupled receptor (Zaballos et al., 1999). The ligand for CCR9 is CCL25 which is expressed by small intestinal epithelial cells in mice and humans (Kunkel et al., 2000), but is not detected in cells within the murine intestinal LP (Stenstad et al., 2007). This indicates that CCL25 diffuses through the LP and becomes embedded on the endothelial vasculature. Adoptive transfer of *Ccr9*^{-/-} CD4⁺ T cells into mice leads to significantly reduced migration of these cells into the SI-LP compared with adoptively transferred wild-type CD4⁺ T cells (Stenstad et al., 2006).

The mechanism of T cell entry into the small intestinal mucosa is likely to proceed through initial tethering and rolling via $\alpha_4\beta_7$ -MAdCAM-1 interactions, allowing interaction between CCR9 and CCL25 which may activate or cluster $\alpha_4\beta_7$ to further enhance

interaction and induce arrest (see Figure 1.6). This is followed by transendothelial migration into the mucosa which remains incompletely understood.

In addition to facilitating migration of lymphocytes into the intestinal LP, CCR9 is involved in recruitment of T cells directly into the epithelium where they are classed as intra-epithelial lymphocytes (reviewed by Agace, 2006). Accordingly, there is a striking reduction in the number of adoptively transferred *Ccr9*^{-/-} CD8⁺ T cells in the small intestinal epithelium of recipient mice as compared with adoptively transferred wild-type CD8⁺ T cells (Johansson-Lindbom et al., 2003, Johansson-Lindbom et al., 2005).

The expression of CCL25 follows a diminishing gradient along the length of the small intestine, with high level expression observed in murine duodenal epithelium and low level expression in ileal epithelium (Stenstad et al., 2007). This indicates that entry of lymphocytes into the LP of the proximal small intestine is more reliant on CCR9-CCL25 signalling. Accordingly, the majority of CD4⁺ and CD8⁺ T cells in the human jejunal mucosa are CCR9⁺ (Kunkel et al., 2000), whilst this proportion falls to 60% in human ileal mucosa and just 20 and 10% in human colonic mucosa for CD4⁺ and CD8⁺ T cells, respectively (Papadakis et al., 2001).

The G protein coupled receptor GPR15 has recently been identified in mice as a putative chemokine receptor mediating recruitment of lymphocytes specifically to the large intestinal mucosa and acting in association with $\alpha_4\beta_7$ (Kim et al., 2013). In contrast with CCR9, GPR15 did not influence recruitment of lymphocytes into small intestinal mucosa. Elevated expression of *GPR15* is also observed in human large intestinal mucosa, suggesting that this system may be shared between mice and humans (Kim et al., 2013).

1:9.3 Migration of IgA⁺ activated B cells into the intestinal mucosa

IgA⁺ activated B cells also home to the small intestinal mucosa in mice through expression of CCR9 (Pabst et al., 2004). Furthermore, the majority of IgA⁺ plasma cells within human intestinal mucosa are $\alpha_4\beta_7^+$ (Farstad et al., 1995). In contrast, migration of murine IgA⁺ B cells to the large intestinal mucosa as well as a range of other mucosal sites is mediated by expression of CCR10, which responds to CCL28 produced by epithelial cells (Pan et al., 2000, Wang et al., 2000).

1:9.4 Migration of effector T cells to extraintestinal sites

In contrast to lymphocytes primed in the GALT and PPs, lymphocytes primed in skin-draining lymph nodes in mice express a range of receptors that facilitate homing into inflamed skin. This includes E-selectin ligands and P-selectin ligands which interact with E-selectin and P-selectin, respectively, expressed on endothelial cells, and CCR4 which interacts with the chemokine CCL17 (reviewed by Sigmundsdottir and Butcher, 2008). In mice, E-selectin and P-selectin expression by cutaneous endothelial cells are required to induce rolling of lymphocytes in the steady state (Weninger et al., 2000).

In mice, RA suppresses the induction of E-selectin ligands whilst enhancing induction of $\alpha_4\beta_7$ and CCR9 (Iwata et al., 2004). Vitamin D receptor, a nuclear receptor which pairs with RXR also induces a 'skin-homing' CCR10⁺ phenotype on T cells during activation *in vitro* whilst suppressing induction of $\alpha_4\beta_7$ and CCR9 (Sigmundsdottir et al., 2007). This allows T cells to home toward CCL27 preferentially expressed in murine inflamed skin (Reiss et al., 2001).

In humans, a significant proportion of T cells present in inflamed and healthy human skin express cutaneous lymphocyte antigen (CLA) (Picker et al., 1990, Clark et al.,

2006), an inducible carbohydrate modification of P-selectin glycoprotein ligand-1 (PSGL-1) (Fuhlbrigge et al., 1997). CLA interacts with E-selectin (Berg et al., 1991) which is a lectin preferentially expressed by human cutaneous endothelial cells during chronic inflammation (Picker et al., 1991). CLA is therefore likely to mediate the migration of circulating human effector T cells into inflamed skin. Furthermore, a significantly greater proportion of effector/memory T cells in human skin draining lymph nodes express CLA⁺ as compared with lymphoid tissue in the appendix, indicating that CLA is induced in humans during T cell activation (Picker et al., 1993). In contrast with mice, treatment of human T cells with both RA and vitamin D result in suppression of CLA expression (Yamanaka et al., 2008).

1:10 RA-mediated regulation of lymphocyte gut homing

The induction of a gut-homing $\alpha_4\beta_7^+$ CCR9⁺ profile on lymphocytes in mice is mediated by exposure of the cells to RA during activation *in vitro* (Iwata et al., 2004). The specialized ability of murine intestinal and MLN CD103⁺ DCs to induce $\alpha_4\beta_7$ and CCR9 expression on lymphocytes (Johansson-Lindbom et al., 2003, Stagg et al., 2002, Jaensson et al., 2008) is therefore dependent on the capacity of these DCs to generate RA. This may be complemented within MLNs by RA produced by stromal cells (Hammerschmidt et al., 2008).

Furthermore, it has been demonstrated in OT-I cells in which RAR signalling induces expression of luciferase that MLN CD103⁺ but not CD103⁻ DCs induce RAR signalling early during a T cell interaction, and prior to the first cell division (Jaensson et al., 2008, Svensson et al., 2008).

Human MLN CD103⁺ but not CD103⁻ DCs induce RA-dependent CCR9 expression on CD8⁺ T cells (Jaensson et al., 2008). This indicates that as in the mouse, CD103⁺ DCs in the MLN have a specialised ability to generate RA. In contrast, both subsets induced $\alpha_4\beta_7$ expression on CD8⁺ T cells. Their ability to imprint gut-homing on CD4⁺ T cells was not addressed in this work.

As discussed in section 1:11, many studies identify an enhanced ability to produce RA through RALDH enzyme activity exclusively in CD103⁺ DCs. However, CD103⁻ DCs collected from afferent lymphatics display equivalent RALDH activity and ability to induce CCR9 expression on naive CD8⁺ T cells (Cerovic et al., 2012). This discrepancy may result from confusion over the earlier 'CD103⁻ DC phenotype', which would certainly contain CD11c⁺ CX₃CR1-GFP^{hi} M Φ which do not possess enhanced RALDH activity (Schulz et al., 2009). By collecting cells from afferent lymphatics, only migratory conventional CD103⁻ DCs are analysed as the CX₃CR1-GFP^{hi} M Φ do not migrate in the steady state (Schulz et al., 2009).

1:10.1 RA-mediated induction of $\alpha_4\beta_7$

The α_4 and β_7 integrin chains are produced independently in cells before binding and moving to the cell surface (reviewed by Hynes, 2002). In murine CD4⁺ and CD8⁺ T cells the α_4 gene (*Itga4*) is under direct transcriptional control by RA whilst the β_7 gene (*Itgb7*) is expressed constitutively (Kang et al., 2011). The basic leucine zipper transcription factor, ATF-like (BATF) facilitates interaction of RAR α with the *Itga4* promoter and is required for the acetylation of histones in the regulatory region of this promoter (Wang et al., 2013). In BATF^{-/-} mice, there is reduced mRNA expression of *Itga4* and surface $\alpha_4\beta_7$, but *Itgb7* mRNA levels are unchanged. This results in reduced numbers of CD4⁺ and CD8⁺ T cells in the colon, SI-LP and PPs (Wang et al., 2013).

In addition to β_7 , α_4 may also combine with β_1 . The $\alpha_4\beta_1$ integrin binds to vascular cell adhesion molecule (VCAM)-1, which is expressed in non-intestinal sites including inflamed skin (reviewed by Agace, 2010). Whilst both β_7 and β_1 act in competition for the binding of α_4 , β_1 is the dominant binding partner to α_4 in mice (DeNucci et al., 2010). The presence of RA during T cell activation therefore results in increased α_4 expression, allowing β_7 to overcome competition from β_1 to create $\alpha_4\beta_7$ at the cell surface. In mice deficient for β_1 , antigen-specific T cells uniformly express $\alpha_4\beta_7$ following infection with *Listeria monocytogenes* and selectively home into PPs (DeNucci et al., 2010).

Beyond RA, induction of $\alpha_4\beta_7$ on OT-I cells by murine CD103⁺ DCs pulsed with OVA peptide is influenced by antigen dose (Svensson et al., 2008). Smaller doses associate with greater induction of $\alpha_4\beta_7$, and enhanced migration of CD8⁺ T cells into the SI-LP following IP administration of OVA to mice. This effect is independent of the level of RA signalling that the CD8⁺ T cell experiences, indicating that additional factors overlap in the regulation of $\alpha_4\beta_7$ expression.

Induction of $\alpha_4\beta_7$ by RA may also be counteracted by vitamin D receptor (VDR) signalling (Sigmundsdottir et al., 2007). This mechanism may involve sequestering of RXR into VDR-RXR complexes, reducing the level of RAR signalling. VDR signalling has also been proposed as an inducer of skin-homing markers such as E-selectin ligands and CCR10

1:10.2 RA-mediated regulation of CCR9

In contrast with $\alpha_4\beta_7$, CCR9 expression facilitates migration of lymphocytes into the SI-LP and epithelium but not into the LI in mice (reviewed by Agace, 2010). Like α_4 , CCR9

is under direct transcriptional control by RA (Kang et al., 2011). Following CD4⁺ and CD8⁺ T cell activation there is increased expression of *Nfatc1* and *Nfatc2*, and both proteins are recruited into the nucleus. NFATc2 promotes *CCR9* expression through enhanced recruitment of RAR α /RXR α to the *CCR9* promoter, whilst NFATc1 suppresses this induction. In the period following T cell activation, nuclear levels of NFATc1 fall whilst nuclear NFATc2 is increased. The presence of RA therefore supports NFATc2-mediated induction of *CCR9* expression, leading to increased surface CCR9 (Ohoka et al., 2011).

Induction of CCR9 on naive CD4⁺ T cells by RA may also be further enhanced by direct stimulation of RXR. Naive OT-II cells adoptively transferred into mice injected IP with ova display enhanced homing into the SI-LP when administered in conjunction with the RXR agonist PA024 (Takeuchi et al., 2010).

Murine naive CD8⁺, but not CD4⁺ T cells express CCR9 on their surface which is lost during stimulation in the absence of additional RA (Svensson et al., 2002). This may indicate a differential relationship between these subsets and the induction of CCR9 by RA. In support of this, CCR9 is efficiently induced on transferred OT-I but not OT-II cells in mice injected IP with OVA (Semmrigh et al., 2011).

Regulation of $\alpha_4\beta_7$ and CCR9 expression in humans is also regulated by exposure to RA (Jaensson et al., 2008). The extent to which the mechanisms of RA-mediated induction for $\alpha_4\beta_7$ and CCR9 in humans compares with mice is however unclear.

1:11 Regulation of RA production by intestinal CD103⁺ DCs

A key feature of murine intestinal CD103⁺ DCs, particularly those found within the small intestine and MLNs, is an ability to generate the vitamin A metabolite retinoic acid (RA)

via expression of retinaldehyde dehydrogenase (RALDH). This enables MLN CD103⁺ DCs to induce a gut-homing $\alpha_4\beta_7^+$ CCR9⁺ phenotype on activated T cells (see section 1:9.2), and can influence the induction of distinct T cell profiles (see section 1:8). RA metabolism and RA-mediated regulation of T cell gut-homing by CD103⁺ DCs are discussed further in sections 1:12 and 1:10, respectively.

Murine CD103⁺ DCs within the SI-LP and MLN possess an enhanced ability to generate RA through the activity of RALDH enzymes, as compared with CX₃CR1-GFP^{hi} resident MΦ in the SI-LP which display 3 fold reduced expression of *Aldh1a2* (Schulz et al., 2009). RALDH activity of intestinal DCs correlates closely with tissue concentration of retinol and RA, which is greatest in the duodenum and decreases along the length of the intestine (Villablanca et al., 2011b). This indicates that exposure of CD103⁺ DCs to RA in the intestinal mucosa may be key to the generation of RALDH activity. Vitamin A-deficient (VAD) mice display significantly reduced RALDH2 activity in their MLN and SI-LP CD103⁺ DCs (Molenaar et al., 2011, Jaensson-Gyllenback et al., 2011). Furthermore, RALDH activity of MLN CD103⁺ DCs is enhanced following oral administration of RA to VAD mice (Villablanca et al., 2011b). Finally, intrarectal administration of RA enhances RALDH activity of colonic CD103⁺ DCs, suggesting that reduced tissue RA is responsible for their relatively diminished RALDH activity (Jaensson-Gyllenback et al., 2011).

The intestinal epithelium may act as a source of RA for the induction of RALDH activity in CD103⁺ DCs. As an important site of uptake of dietary retinol (discussed further in section 1:12), small intestinal enterocytes but not LP cells express cellular retinol binding protein II (CRBP II) (Crow and Ong, 1985). SI-LP CD103⁺ DCs from CRBP II^{-/-} mice have significantly reduced RALDH activity which suggests that the ability of

enterocytes to contain and process retinol in some way is critical for the subsequent induction of RALDH activity in CD103⁺ DCs (McDonald et al., 2012).

Retinoids derive from the diet (see section 1:12), and their persistence within the intestinal lumen is prolonged through their presence in bile salts which further support induction of RALDH activity in murine CD103⁺ DCs (Jaensson-Gyllenback et al., 2011, McDonald et al., 2012).

The upregulation of RALDH activity in murine CD103⁺ DCs is rapid given the absence of activity in circulating pre-DCs (Jaensson-Gyllenback et al., 2011) and the relatively fast turnover of these cells within the LP compared with resident CX₃CR1-GFP^{hi} MΦ (Rivollier et al., 2012, Schulz et al., 2009). The inability of CX₃CR1-GFP^{hi} MΦ to upregulate RALDH activity to the same extent as CD103⁺ DCs despite persisting in the tissue for long periods of time (Schulz et al., 2009) could indicate the presence of negative RALDH factors which restrain this activity in some cells. Indeed, prostaglandin E₂ (PGE₂) suppresses the induction of RALDH activity in both murine and human monocyte-derived DCs via induction of cyclic adenosine monophosphate (cAMP)-regulated proteins (Stock et al., 2011). Interestingly, MLN DCs have reduced expression of the E-prostanoid-2 (EP2) receptor, suggesting that RALDH activity develops through absence of restriction by PGE₂ signalling. Whether suppression of RALDH activity in CX₃CR1-GFP^{hi} MΦ is mediated through PGE₂ remains to be determined.

RA alone is sufficient to induce *Aldh1a2* expression and RALDH activity in murine splenic DCs, and this induction is blocked with inhibition of the mitogen associated protein kinase (MAPK) ERK (Villablanca et al., 2011b). Furthermore, mice fed an oral ERK antagonist display reduced RALDH activity in their MLN CD103⁺ DCs, indicating

that this pathway is important in the *in vivo* induction of RALDH activity by RA in the intestine (Villablanca et al., 2011b). Induction of RALDH activity in splenic DCs is also reduced in MyD88^{-/-} mice, suggesting an involvement of TLR signalling in this process (Villablanca et al., 2011b). Consistent with this view, stimulation of murine splenic DCs with the TLR1/2 agonist Pam3CSK4 (a bacterial lipoprotein) of the fungal TLR2 ligand zymosan, is sufficient to induce RALDH activity (Wang et al., 2011b). This process requires JNK/MAPK signalling, as opposed to ERK/MAPK, suggesting that JNK and ERK may regulate TLR and RA-induced *Aldh1a2* expression, respectively. In contrast, stimulation of murine splenic DCs with the fungal TLR2 ligand zymosan induces RALDH activity in a ERK-dependent manner (Manicassamy et al., 2009), suggesting further complexity. TLR2^{-/-} mice also display reduced RALDH activity of MLN CD103⁺ DCs, with reduced imprinting of $\alpha_4\beta_7$ and CCR9 on CD8⁺ T cells (Wang et al., 2011b). This further implicates microbial recognition through TLR1/2 in the development of RALDH activity in intestinal CD103⁺ DCs.

Culturing murine splenic DCs with the zymosan induces expression of CD137 (also known as 4-1BB), a member of the TNF receptor superfamily. Stimulation of this receptor enhances induction of RALDH activity in these cells in an ERK-dependent manner (Lee et al., 2012). Furthermore, the majority of murine MLN CD103⁺ DCs are CD137⁺ (Lee et al., 2012), indicating that this pathway may also be active in promoting upregulation of RALDH activity *in vivo*.

Infection of mice with adenovirus 5 leads to gut-homing of antigen-specific CD8⁺ T cells. This is caused by GM-CSF-mediated upregulation of RALDH activity in non-intestinal DCs, leading to the induction of $\alpha_4\beta_7$ on CD8⁺ T cells (Ganguly et al., 2011a). GM-CSF also induces *Aldh1a2* expression in murine bone marrow (BM)-derived DCs, and mice deficient in GM-CSF display significantly reduced RALDH activity in intestinal

DCs (Yokota et al., 2009). The DC-specific cytokine Flt3L alone did not induce *Aldh1a2* expression in BM-DCs, but did in the presence of either IL-4 or IL-13 (Yokota et al., 2009). The factors influencing RALDH activity of murine intestinal CD103⁺ DCs are summarized in Figure 1.7.

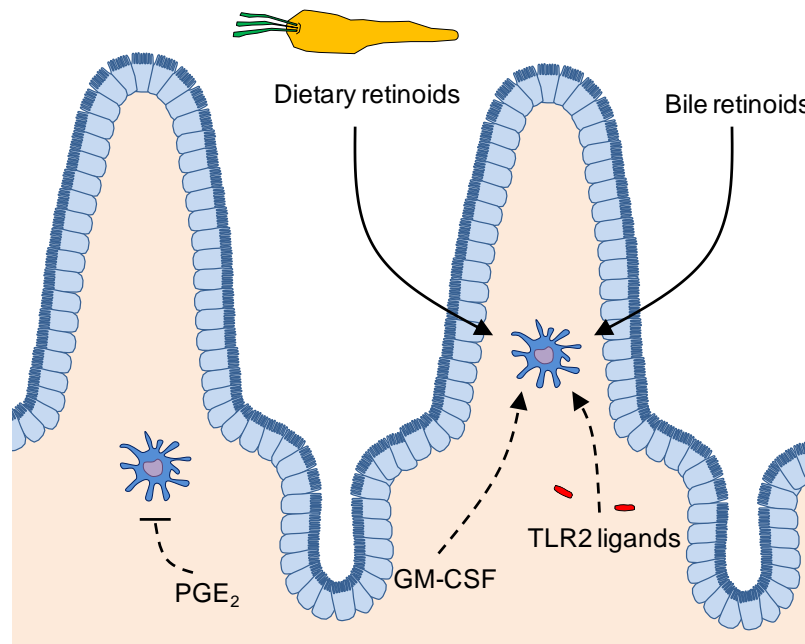


Figure 1.7: Factors regulating RALDH activity of murine SI-LP CD103⁺ DCs. Retinoids derived from the diet and present within bile provide the main signals promoting RALDH activity within murine SI-LP CD103⁺ DCs. GM-CSF and TLR2 ligands such as zymosan induce RALDH activity in murine splenic DCs *in vitro* and so may also influence RALDH in intestinal CD103⁺ DCs. PGE₂ is a negative regulatory of RALDH in DCs; it may suppress RALDH activity in cells other than CD103⁺ DCs.

1:12 RA metabolism

RA is derived from dietary vitamin A (retinol), ingested as retinyl esters from animal tissue and carotenoids from vegetables. Most retinyl esters are enzymatically converted to retinol in the intestinal lumen before being absorbed by enterocytes. Some carotenoids are converted to retinol within the enterocytes (Blomhoff et al., 1990).

Retinol is then converted back into retinyl esters and packaged into chylomicrons which enter circulation via the lymphatics and are processed in the liver. A steady release of retinol associated with retinol binding protein (RBP) into the circulation ensures that retinol is distributed to target sites around the body (Blomhoff et al., 1990).

Transfer of retinol into cells occurs either via the transporter STRA6 (Kawaguchi et al., 2007), or by passive diffusion. Inside the cell, retinol is bound by cellular retinol binding protein (CRBP)-I and is converted into retinoic acid by a two stage oxidation process. The first is reversible, catalysed predominantly by the microsomal short chain dehydrogenase/reductase enzymes (SDR) and generates retinal. Well established members of this enzyme family include RDH10 (Rdh1 in mice), RDH16 and DHRS9. Retinal is then irreversibly oxidised to RA by members of the cytosolic retinaldehyde dehydrogenase (RALDH) family, of which RALDH1 (ALDH1A1), RALDH2 (ALDH1A2) and RALDH3 (ALDH1A3) are the best studied. Whilst both oxidations require the cofactor nicotinamide adenosine dinucleotide (NAD), the first stage is rate limiting in the presence of CRBP-I (reviewed by Napoli, 2012). The production of RA is summarized in Figure 1.8.

Different retinoid isoforms exist based on whether the functional groups of the molecule are on different sides (*trans*) or on the same side (*cis*) of the four double bonds present in the side chain of the structure. The *all-trans* form predominates, and is the most biologically active. The different enzymes have varying affinities for 9-*cis* and *all-trans* retinoids, but an *in vivo* role for 9-*cis* RA remains controversial (reviewed by Napoli, 2012).

The net level of RA within a cell is determined by the rate of degradation as well as synthesis. The cytochrome P450 (CYP)26 family of enzymes are known to convert RA

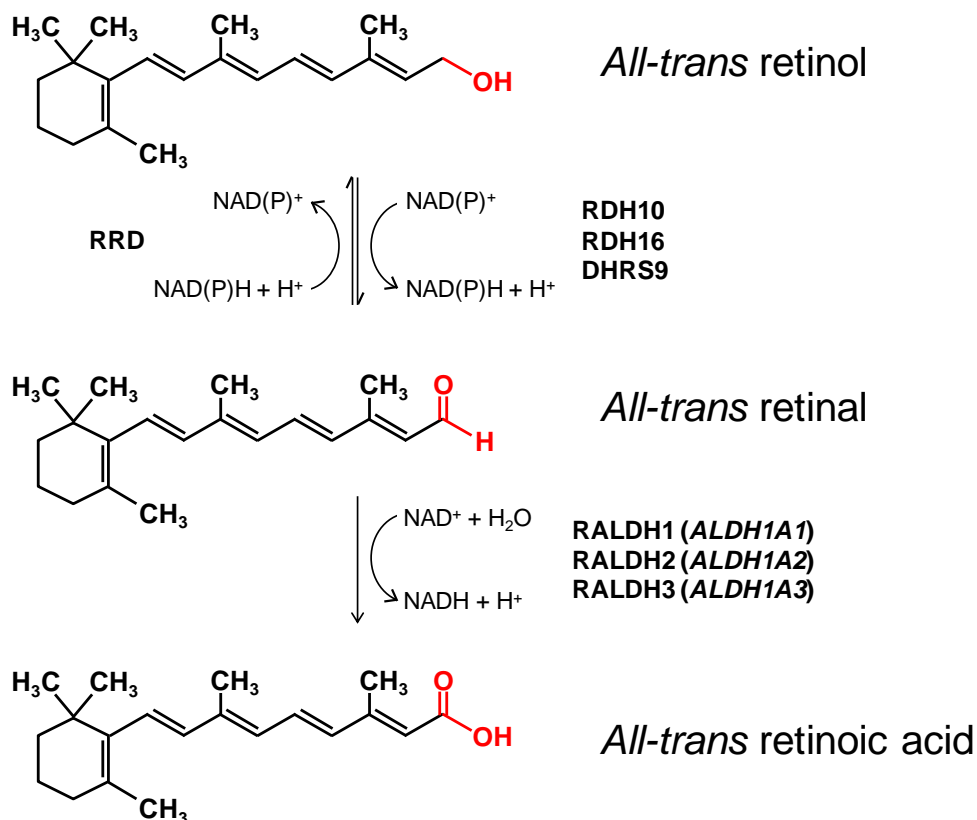


Figure 1.8: Generation of RA from retinol. All-trans retinol is derived from dietary β -carotene and retinyl esters e.g. retinyl palmitate. This is reversibly oxidised by members of the short chain dehydrogenase/reductase (SDR) family of enzymes including RDH10, RDH16 and DHRS9. Retinol reductase enzymes (RRD) catalyse the reduction of retinal back to retinol. Retinal is then irreversibly oxidised to retinoic acid by the ALDH1A (RALDH) family of enzymes including RALDH1, RALDH2 and RALDH3.

into the polar and much less biologically active metabolites 4-hydroxy RA and 4-oxo RA (Niederreither and Dolle, 2008). Members of this family include CYP26a1 and CYP26b1, which has been implicated in regulating CCR9 expression in murine T cells (Takeuchi et al., 2011).

1:13 RA signalling

The retinoic acid receptor (RAR) exists as 3 isoforms (α , β , and γ) and forms a heterodimer with the one of the 3 isoforms of the retinoid X receptor (RXR; α , β , and γ).

RAR/RXR is a member of the superfamily of nuclear receptors, and is a ligand-activated transcription factor. In absence of RA, RAR/RXR recognises and occupies a short sequence of DNA known as a RA response element (RARE) and associates with transcription co-repressors. These include histone deacetylases and methyl transferase complexes that render the DNA inaccessible. Binding of RA to RAR/RXR leads to a conformational shift in a ligand binding domain, releasing co-repressors and recruiting activators involved in chromatin remodelling and activation of transcription machinery (Niederreither and Dolle, 2008).

RA is associated with cellular retinoic acid binding proteins (CRABPs), which mediate transfer of RA onto RAR/RXR (Napoli, 2012). Alternative binding proteins such as fatty acid binding protein (FABP)-5 have been associated with transfer of RA to separate nuclear receptors such as PPAR γ (Schug et al., 2007).

Hundreds of genes have been identified as being regulated by RA. However, only relatively few have been shown to possess RARE indicating direct regulation (Balmer and Blomhoff, 2002). This implies the vast majority of RA-regulated genes are regulated in an indirect manner.

1:14 Inflammatory bowel disease

1:14.1 General features of IBD

Patients with inflammatory bowel disease (IBD) experience relapsing and remitting episodes of intestinal inflammation. This has a significantly detrimental impact on quality of life, with symptoms including abdominal pain, diarrhoea with blood and/or mucus, vomiting and nausea. IBD is believed to be the result of a complex interplay between an exaggerated immune response to the commensal microbiota, genetic

predisposition and a range of environmental factors which are not fully understood (reviewed by Baumgart and Carding, 2007).

IBD is divided into two separate diseases, Crohn's disease (CD) and ulcerative colitis (UC). Early inflammatory events in CD typically manifest as ulcers overlying lymphoid follicles and PPs. Inflammation affects multiple layers of the intestine (transmural), extending beyond the submucosa as far as the serosa. Isolated areas are affected and may be surrounded by apparently normal mucosa (focal inflammation). Furthermore, inflammation may affect any part of the gastrointestinal tract (reviewed by Day and Morson, 2003). A cohort study of 306 CD patients in the US identified inflammation in the TI in 45%, the colon in 32% and both the TI and colon (ileocolonic) in 18.6 % of patients at diagnosis (Thia et al., 2010). Patients may develop stricturing where the intestine narrows causing bowel obstruction, or fistulae where transmural inflammation forms a passageway into a new area of the body (e.g. between the colon and skin). The cumulative risk for developing either a stricturing or penetrating disease 20 years after diagnosis is approximately 50% (Thia et al., 2010). Non-caseating granulomas are present in approximately 50% of CD patients, and may be located in the mucosa, submucosa and in lymphoid tissue (reviewed by Day and Morson, 2003). The varying location and severity of inflammation as well as the presence or absence of stricturing fistulae in CD patients demonstrates the highly heterogeneous nature of this disease.

Inflammation in UC is present almost universally in the rectum, and extends to varying extents along the large intestine in a continuous manner. Where the entire large intestine is inflamed (pan colitis), inflammation may also be present in the TI. Furthermore, the inflammation is more superficial than in CD, largely limited to the mucosa and submucosa (reviewed by Day and Morson, 2003). Pathological features of UC include leukocyte infiltration into the LP. Mucosal architecture also becomes

distorted with a shortening of crypts, mucin depletion and ulceration (reviewed by Ordas et al., 2012).

There are a number of associations between IBD and additional extra-intestinal manifestations. For example, there is increased incidence in arthropathy and the skin conditions pyoderma gangrenosum and erythema nodosum in both CD and UC patients (reviewed by Day and Morson, 2003). There is also an association between liver pathology and chronic UC. Between 5 and 10 % of UC patients develop primary sclerosing cholangitis (PSC), where fibrosis causes inflammation and obstruction of the bile ducts (cholangitis). Up to 70% of PSC patients also have UC (reviewed by Day and Morson, 2003).

1:14.2 Genetic predisposition to IBD

IBD manifests in genetically susceptible individuals, however the nature of this susceptibility is complex and known genetic variants account for only approximately 13.6 % of CD cases and 7.5 % of UC cases (Jostins et al., 2012).

Early studies identified frameshift and missense variants in nucleotide oligomerization domain (NOD)-2 in CD patients (Hugot et al., 2001, Ogura et al., 2001). NOD2 acts as an intracellular receptor for muramyl dipeptide (MDP), a component of bacterial peptidoglycan, leading to activation of NF- κ B and the production of pro-inflammatory cytokines (Inohara et al., 2003, Girardin et al., 2003). Human monocyte-derived DCs (MoDCs) derived from CD patients with *NOD2* mutations display a significantly reduced ability to process bacterial antigen and to present bacteria-derived peptides (Cooney et al., 2010). These genetic studies therefore underscore the importance of ability of the intestinal innate immune system to respond to bacterial encroachment appropriately.

Genome wide association studies (GWAS) have identified 163 genetic loci implicated in IBD (Jostins et al., 2012). Of these 163 loci, 30 are specific for CD and 23 are specific for UC, and so the majority are common to both diseases (Jostins et al., 2012). Whilst these loci refer to specific areas of the genome, the identity and function of the genes within these areas are not always known (reviewed by Graham and Xavier, 2013). The magnitude of risk associated with each polymorphism is very small, but provides a potential insight into the particular pathways that may be involved in the pathogenesis of IBD. This includes autophagy, a lysosomal degradation pathway whereby cell components are degraded for re-use, and by which internalized bacteria may also be degraded (reviewed by Levine and Kroemer, 2008). Variants in *ATG16L1* are implicated in CD through impaired Paneth cell function, as with diminished autophagy these highly secretory cells are less able to manage an accumulation of misfolded protein (Cadwell et al., 2008). This may lead to increased bacterial encroachment due to reduced output of anti-microbial peptides. In addition, stimulation of NOD2 promotes recruitment of ATG16L1 to the cell membrane for the internalization of bacteria and subsequent destruction in the autophagosome (Travassos et al., 2010).

In contrast to CD, NOD2 variants are not associated with UC (Anderson et al., 2011). UC-specific variants include *CDH1* which encodes E-cadherin and is expressed by intestinal epithelial cells (Anderson et al., 2011). In addition, variants in the region encoding *HNF4A* have been associated with UC (Barrett et al., 2009). *HNF4A* encodes the transcription factor hepatocytes nuclear factor 4 which regulates expression of tight junction proteins in epithelium of the liver and intestine (Battle et al., 2006). This specifically implicates the barrier function of the intestinal epithelium in UC as a source of predisposition to disease.

Both CD and UC are associated with variants in genes associated with T_H17-mediated immunity, including *IL23R*, *IL12B* (encoding IL-12/23p40), *JAK2* and *STAT3* (Anderson et al., 2011). Furthermore, both CD and UC are associated with variants in *IL10* (Franke et al., 2008, Franke et al., 2010). Overall therefore, individuals with IBD are genetically predisposed to some extent to have a dysregulated immune response which may be due to genetically predisposed barrier function as in UC, or a genetically predisposed inability to cope with bacterial encroachment as in CD. As described previously, known genetic variants account for only approximately 13.6% of CD cases and 7.5% of UC cases (Jostins et al., 2012). One study observed that in a group of monozygotic twins where at least one sibling had CD, the second also had CD in 35 % of cases compared with just 3 % for dizygotic twins. For UC, the figures were 16 and 2 %, respectively (Spehlmann et al., 2008). Whilst this indicates a clear role for genetic pre-disposition in both CD and UC, the fact that in the majority of cases only one of the identical twins had IBD indicates the role that additional environmental factors play in the pathogenesis of disease. It has been proposed that epigenetics, or non-coding DNA modifications that influence gene expression and may be mitotically inherited bridge the 'missing heritability' gap between known disease variants and environmental factors (reviewed by Ventham et al., 2013).

1:14.3 Environmental factors

IBD incidence varies according to geographical location, with high incidence areas including North America, northern Europe and Australia. Low incidence areas include southern and eastern Europe, Asia and many developing countries. The incidence of IBD is however steadily increasing in these low incidence areas (reviewed by Ng et al., 2013). This variation has been linked to a range of environmental factors associated

with the 'western lifestyle' including urbanisation of society, improved sanitation, diet, antibiotic use, microbial exposure and pollution (reviewed by Ng et al., 2013). Tobacco smoking is a clearly defined environmental factor associated with modifications in IBD incidence. Smoking is associated with increased severity of CD (Seksik et al., 2009), and is protective against UC (Mahid et al., 2006).

IBD is believed to result from a breakdown in the relationship between intestinal microbiota and the host immune system (reviewed by Baumgart and Carding, 2007). An important environmental factor in IBD is therefore the nature of the microbiota itself. IBD associates with reduced proportions of Bifidobacteria, Lactobacilli, Bacteroides and Firmicutes (reviewed by Sartor, 2008). An increased prevalence of an adherent invasive *E. coli* (AIEC) has been observed specifically in ileal CD and may contribute to disease pathogenesis through invasion of the intestinal epithelium (Boudeau et al., 1999, Darfeuille-Michaud et al., 2004, Martin et al., 2004b). Furthermore, a reduction in the numbers of the Firmicute *Faecalibacterium prausnitzii* associates with increased risk of post-operative CD recurrence (Sokol et al., 2008b). There is also a significant association between acute gastrointestinal infection and the onset or exacerbation of IBD (García Rodríguez et al., 2006, Porter et al., 2008, Gradel et al., 2009). This is consistent with infection triggering a breakdown in the host-microbiota relationship in a subset of genetically susceptible individuals.

As discussed in section 1:4.2, the intestinal epithelium is required to provide a physical barrier between the luminal microbiota and the interior of the mucosa. Increased intestinal permeability is associated with both CD and UC, indicating that the intestinal immune system is exposed to microbiota-derived antigen to a significantly greater extent in IBD patients compared with healthy controls (reviewed by Baumgart and Sandborn, 2012, Ordas et al., 2012). The structure of the epithelium itself is modified in

CD, which reduced expression of tight junction proteins in inflamed areas (Zeissig et al., 2007).

In CD, reduced expression of the mucin gene *MUC1* is observed in the inflamed ileum of CD patients (Buisine et al., 1999), indicating that defective mucin production may contribute to bacterial encroachment. Furthermore, a genetic variant of *MUC1* is associated with CD (Franke et al., 2010). Active UC associates with reduced production of MUC2 (Van Klinken et al., 1999), supporting defective separation of the intestinal microbiota from the interior of the mucosa in the pathogenesis of disease. As discussed in section 1:4.1, *Muc2*^{-/-} mice display bacterial encroachment directly onto the large intestinal epithelium (Johansson et al., 2008), associating with the onset of colitic symptoms including diarrhoea, rectal prolapse as well as severe growth retardation (Van der Sluis et al., 2006).

1:14.4 Immune-mediated inflammation in IBD

As described in section 1:12.1, IBD is believed to result from an inappropriate immune response to commensal microbiota in genetically susceptible individuals. Inflammation in the intestine is driven by a range of pro-inflammatory mediators including cytokines with a broad range of effects. The prototypical pro-inflammatory cytokine is TNF α , which is produced in significantly elevated quantities by mononuclear cells, including M Φ and T cells, in the inflamed mucosa of both CD and UC patients compared with healthy controls alongside IL-6 and IL-1 β (Reinecker et al., 1993). Subepithelial M Φ are the predominant source of TNF α in UC, whilst TNF α ⁺ cells are evenly distributed throughout the LP in CD and beyond into the submucosa where they cluster around arterioles and venules (Murch et al., 1993). This distribution of TNF α ⁺ cells therefore

parallels the differing nature of intestinal inflammation between UC and CD patients as discussed in section 1:12.1.

Therapies which specifically target TNF α such as the neutralising monoclonal antibodies infliximab and adalimumab are significantly more effective in inducing clinical remission in patients with active CD (reviewed by Akobeng and Zachos, 2004) and UC (reviewed by Lawson et al., 2006) than placebo. However, the soluble TNF receptor-Fc fusion protein etanercept is ineffective in the treatment of CD (Sandborn et al., 2001). Whilst both infliximab and etanercept effectively neutralise soluble TNF α , Infliximab alone is able to bind surface TNF α on activated T cells and induce apoptosis in peripheral blood and intestinal LP activated T cells (ten Hove et al., 2002, Van Den Brande et al., 2003). Infliximab also induces apoptosis in blood monocytes from CD patients (Lugering et al., 2001). As discussed in section 1:15.5, human CD14⁺ monocytes are implicated in the pathogenesis of IBD. Overall, this indicates that neutralisation of soluble TNF α is insufficient to resolve intestinal inflammation in IBD, which instead requires removal of cells which are important sources of TNF α and other pro-inflammatory cytokines.

As discussed in section 1:14.5.3, over-expression of TNF α in mice leads to inflammation of the ileum with several features of human CD (Kontoyiannis et al., 1999). A key target of TNF α signalling in this model are intestinal myofibroblasts, which are stromal cells in the LP, and respond by producing a range of matrix metalloproteinases (MMPs) which degrade the tissue and propagate inflammation (Armaka et al., 2008).

Inflammation in CD is associated with high levels of production of IFN γ by LP T cells (Fuss et al., 1996), and antigen presenting cells in CD patients produce greater

amounts of IL-12 compared with UC patients and healthy controls (Hart et al., 2005, Fuss et al., 2006). There is also an accumulation of Tbet⁺ T cells in the inflamed intestinal LP of CD patients compared with UC and healthy controls (Neurath et al., 2002). Such observations led to the classification of CD as a T_H1-mediated disorder (reviewed by Strober and Fuss, 2011). Elevated IFN γ is likely to contribute to disease pathogenesis through a diverse range of activities including the activation of endothelial cells promoting recruitment of neutrophils and monocytes into the inflammatory foci, activation of neutrophils and M Φ , leading to the production of further pro-inflammatory cytokines, ROS and RNI, as well as upregulation of MHC molecules and promotion of antigen presentation (reviewed by Boehm et al., 1997).

However, monoclonal antibodies to IFN γ are not effective in inducing a clinical response in CD patients when compared with placebo, suggesting that whilst IFN γ may contribute to disease pathogenesis, transient reduction in IFN γ is not sufficient to halt an ongoing intestinal inflammatory response (Reinisch et al., 2006, Reinisch et al., 2010).

Increased expression of IL-17A by intestinal LP T cells is observed in both CD and UC patients compared with healthy controls (Fujino et al., 2003). This has led to the proposal that inflammation in CD is mediated by a mixed T_H1/T_H17 response (reviewed by Strober and Fuss, 2011). IL-17A may contribute to intestinal inflammation as it synergizes with TNF α in inducing the production of the neutrophil chemokines IL-8 (CXCL8) and CXCL1 by human intestinal epithelial cells *in vitro* (Lee et al., 2008). A population of IFN γ ⁺/IL-17A⁺ T cells is present in peripheral blood and inflamed intestinal LP in CD patients which produces both IFN γ and IL-17A upon stimulation with IL-23 (Kleinschek et al., 2009). In contrast, peripheral blood IFN γ ⁺/IL-17A⁺ T cells in healthy controls require prior activation with IL-1 β in order to respond to IL-23 with IFN γ and IL-

17A production (Kleinschek et al., 2009). The role of IL-17A in the pathogenesis of CD remains controversial however as monoclonal antibodies against IL-17A are ineffective in CD, and associate with increased susceptibility to bacterial and fungal infections (Hueber et al., 2012). This suggests that IL-17A may exert both protective as well as pro-inflammatory effects in CD. This is supported by the T cell transfer colitis mouse model of intestinal inflammation (see section 1:12.5.2) whereby adoptive transfer of T cells from *Il17a*^{-/-} mice into *Rag*^{-/-} recipients causes a more severe intestinal inflammation than transfer of WT cells (O'Connor et al., 2009). Furthermore in the DSS colitis mouse model of intestinal inflammation (see section 1:12.5.1) *Il17a*^{-/-} mice display a more severe colitis upon administration of DSS than WT mice (Yang et al., 2008b). In contrast, in *Il17f*^{-/-} mice this colitis is attenuated (Yang et al., 2008b), indicating contrasting protective and inflammatory roles within the IL-17 cytokine family.

The transition from normal mucosa to macroscopically inflamed mucosa in the neo TI of CD patients following surgical removal of the original TI is associated with abundant levels of TNF α and IFN γ alongside a marked increase in IL-17A, IL-6 and IL-23 expression (Zorzi et al., 2013). Established lesions contain a mixed T_H1/T_H17 response, with relatively low levels of TNF α (Zorzi et al., 2013).

High levels of IL-6 and IL-1 β produced by stromal cells in the intestinal mucosa of CD patients compared with healthy controls promote the proliferation of T cells *in vitro*, whilst IL-1 β enhances production of both IFN γ and IL-17A (Huff et al., 2011).

UC is associated with elevated concentrations of the T_H2 cytokines IL-13 and IL-5, but not elevated expression of the classic T_H2 cytokine IL-4 (Fuss et al., 1996, Fuss et al., 2004). Such observations led to UC being defined as a 'T_H2-like' immunopathology (reviewed by Strober and Fuss, 2011), although this remains controversial. It has been

proposed that IL-13 produced by a lymphocyte which shares features of NK and T cells (NK T cell) damages the intestinal epithelium in UC through modulation of tight junction proteins (Heller et al., 2005). Recent studies however have indicated that CD4⁺ T cells in both CD and UC display elevated production of IFN γ and IL-17A as compared with healthy controls (Rovedatti et al., 2009). Furthermore, the overall gene expression in CD and UC is remarkably similar, with a rare exception being elevated expression of IL-23 in UC relative to CD intestinal mucosa (Granlund et al., 2013). This therefore challenges the notion that CD and UC are distinguished by the nature of the ongoing adaptive immune response, and indicates that distinct initiation events converge on a T_H1/T_H17-mediated intestinal inflammation.

Inflammation associated with IBD may be the result of a failure of T_{REG} populations within the intestinal mucosa to restrict the activity of effector T cells. However, identifying T_{REG} within the human intestinal mucosa is made difficult by the transient expression of FOXP3 in activated T cells (Gavin et al., 2006, Allan et al., 2007, Pillai et al., 2007, Merlo et al., 2008). Regardless, CD25^{hi} FOXP3⁺ T cells are present at low density in the intestinal mucosa of healthy controls, and at increased densities in UC and CD patients (Makita et al., 2004, Maul et al., 2005, Uhlig et al., 2006). CD4⁺ CD25^{hi} T cells obtained from the colonic mucosa of healthy controls and IBD patients also suppress the proliferation of autologous peripheral blood T cells (Makita et al., 2004, Kelsen et al., 2005, Maul et al., 2005), consistent with a T_{REG} phenotype. The presence of putative T_{REG} populations within the inflamed mucosa of IBD patients indicates that these cells are unable to suppress effector T cells within this context. This is supported by observations that effector T cells in the inflamed mucosa of IBD patients express SMAD7, and so are resistant to TGF- β -mediated suppression by T_{REG} (Fantini et al., 2009). SMAD7 prevents TGF- β -dependent formation of SMAD2 and SMAD3,

preventing association with SMAD4 and nuclear accumulation and transcriptional activity of SMAD2/3 (Hayashi et al., 1997, Nakao et al., 1997).

Inflamed intestinal mucosa of CD patients contains a population of FOXP3⁺ CD4⁺ T cells with the capacity to produce IL-17A, and which are not observed in UC patients or healthy controls (Hovhannisyan et al., 2011). These FOXP3⁺ IL-17A⁺ cells display features overlapping T_{REG}, T_H17 and T_H1 phenotypes as they suppress proliferation of peripheral blood CD4⁺ T cells *in vitro*, express IL-17A and ROR γ t, as well as expressing IFN γ and Tbet (Hovhannisyan et al., 2011). However these cells are not observed in peripheral circulation, and so may be induced within the intestinal mucosa under the influence of local mediators specific to CD patients. Treatment of CD4⁺ T cells from UC patients but not healthy controls with TGF- β is sufficient to induce IL-17A production from FOXP3⁺ T cells, suggesting that TGF- β as well as other factors induce this phenotype in CD (Hovhannisyan et al., 2011). It is unclear to what extent these distinct cell phenotypes negate each other, and whether overall they exert a pro or anti-inflammatory effect.

1:14.5 Mouse models of IBD

Due to the limitations of studying disease mechanisms in humans, a diverse array of experimental mouse models of IBD have been generated and continue to provide key insights into how inflammation alters cellular behaviour within the intestinal mucosa (reviewed by Strober et al., 2002). This section will provide an overview of the models that have been used to conduct work relevant to the subject of this thesis.

1:14.5.1 *DSS colitis*

Dextran sodium sulphate (DSS) is a sulphated polysaccharide that when given to mice in drinking water induces inflammation in the large intestine within 7 days. This is associated with increased intestinal permeability, increasing exposure of the interior of the intestinal mucosa to intestinal microbiota. Inflammation manifests in a loss of crypts, infiltration of inflammatory cells including neutrophils and MΦ into the mucosa and submucosa, oedema of the submucosa and ulceration (Okayasu et al., 1990). The molecular weight of DSS influences inflammation, with 5 kDa DSS primarily affecting the cecum and proximal colon in contrast with 40 kDa DSS which produces a more severe inflammation of the distal colon (Kitajima et al., 2000). The cytokine profile of DSS colitis is dominated by TNFα and IL-6. Inflammation is also induced in *Rag2*^{-/-} mice which lack T and B cells, indicating that this is a model of innate inflammation in which MΦ and neutrophil-derived pro-inflammatory cytokines play a key role in promoting tissue damage (Dieleman et al., 1994). DSS colitis may also be induced in germ-free mice, indicating that the presence of intestinal inflammation is not absolutely required for the onset of inflammation (Bylundfellenius et al., 1994, Kitajima et al., 2001).

1:14.5.2 *T cell transfer colitis*

The CD45RB^{hi} subset of CD4⁺ T cells is enriched for naive CD4⁺ T cells. Adoptive transfer of these cells into immunodeficient mice (*Rag*^{-/-}, lacking mature T and B cells) induces a severe colitis within 3 to 5 weeks. Inflammation is restricted to the large intestine, and associates with ulceration, epithelial hyperplasia and a loss of mucus production. There is extensive infiltration of mononuclear cells (primarily CD4⁺ T cells and MΦ), with increased production of IFNγ and TNFα which is consistent with a T_H1-dominated T cell response (Powrie et al., 1994b, Powrie et al., 1994a). Administration

of neutralising antibodies to either IFN γ or TNF α significantly attenuates disease (Powrie et al., 1994b). Furthermore, T cells from mice deficient in the T_H1 transcription factor Tbet fail to induce inflammation in the T cell transfer model (Neurath et al., 2002).

Co-transfer of CD45RB^{lo} CD4⁺ T cells which contains mature T cells suppresses the induction of colitis. This is attributed to the presence of CD25⁺ regulatory T cells (T_{REG}) within this subset that restrict activation and expansion of IFN γ ⁺ CD4⁺ T cells via TGF- β and/or IL-10 production (reviewed by Strober et al., 2002). The microbiota plays a key role in the induction of T cell transfer colitis as the severity of inflammation is significantly attenuated in mice with reduced intestinal bacteria (Aranda et al., 1997). The T_H1-mediated immune response of T cell transfer colitis resembles CD in humans, whilst restriction of inflammation to the large intestine parallels inflammation in UC. T cell transfer colitis is associated with a greater number of transcriptional changes that are observed in human IBD as compared with DSS colitis (te Velde et al., 2007).

In a slightly modified version of T cell transfer colitis, naive CD4⁺ T cells are adoptively transferred into *Cd3e* ^{$\Delta 5/\Delta 5$} mice which lack T cells but not B cells (Malissen et al., 1995), leading to rapid expansion of IFN γ ⁺ T cells in the large intestine and chronic inflammation typified by weight loss, diarrhoea and rectal prolapse (Martin et al., 2004a).

1:14.5.3 *TNF Δ ARE ileitis*

Expression of the pro-inflammatory cytokine TNF α is regulated at multiple levels, but critically at the level of mRNA stability. This is determined by a region in the 3' untranslated region (UTR) of the TNF α mRNA which contains adenosine-uracil (AU) multimers which together form AU rich elements (ARE). By removing these ARE the

TNF α mRNA is stabilised, leading to increased production of TNF α protein by both M Φ , lymphocytes, as well as non-haematopoietic cells including synovial fibroblasts (Kontoyiannis et al., 1999). TNF Δ ARE/ Δ ARE mice spontaneously develop intestinal pathology resembling CD at 2 to 4 weeks of age. Inflammation primarily affects the TI, but may also occur in the proximal colon. This includes villous blunting and broadening, mucosal and submucosal infiltration of mononuclear leukocytes, plasma cells and neutrophils, as well increased numbers of lymphoid aggregates and follicles. Over time, inflammation extends deep into the muscular layer of the intestinal wall, reflecting the transmural inflammation of CD. Older mice also display rudimentary granulomata which resemble the non-caseating granulomas often observed in CD.

In addition to intestinal pathology, TNF Δ ARE/ Δ ARE mice also develop severe inflammatory arthritis. Interestingly, intestinal inflammation is significantly suppressed in TNF Δ ARE/+ mice crossed onto a RAG-1 $^{-/-}$ background, indicating a critical role for T and B cells in intestinal inflammation. In contrast, this has no effect on arthritic pathology which indicates a different mechanism of pathogenesis. This may relate to the observed production of TNF α by synovial M Φ in TNF Δ ARE mice (Kontoyiannis et al., 1999).

1:15 Role of mononuclear phagocytes in the pathogenesis of IBD

As initiators of the adaptive immune response in IBD, intestinal DCs could potentially play a pivotal role in the pathogenesis of disease through driving imbalanced immune responses. This section will focus on the contribution of intestinal APC populations including CD103 $^{+}$ DCs in the pathogenesis of IBD.

1:15.1 Murine intestinal CD103⁺ DCs in models of IBD

In the T cell transfer model of colitis (as discussed in section 1:12.5.2), murine CD103⁺ DCs within MLNs have significantly reduced expression of *Aldh1a2* compared with their steady-state counterparts, indicating a reduced ability to produce RA (Laffont et al., 2010). In addition, they have reduced expression of *Tgfb2*, associating with a reduced ability to induce Foxp3⁺ T_{REG} from naive CD4⁺ T cells. Beyond this loss of regulatory properties, they also produce more IL-12p35 and induce greater production of IFN γ and IL-17A by naive CD4⁺ T cells (Laffont et al., 2010). This indicates that in this model, modifications to CD103⁺ DC behaviour including a reduced ability to make RA may contribute to the pathogenesis of disease. It is however difficult to determine whether these modulations are caused by the presence of intestinal inflammation as opposed to being the cause of inflammation.

MLN CD103⁺ DCs in mice with control and inflamed intestinal mucosa express the Flt3L receptor (CD135), indicating that they are following a similar process of differentiation (as discussed in section 1:17.2). This suggests that their origins are constant before and during inflammation, but their behaviour is modified. BrdU experiments also indicate that the turnover of MLN CD103⁺ DCs is more rapid during inflammation (Laffont et al., 2010). If the ability to produce RA is the result of conditioning within the intestinal mucosa, CD103⁺ DCs may spend less time within this environment in inflammation before migrating onto the MLNs.

In the TNF Δ ARE model of ileitis (see section 1:14.5.3) murine CD103⁺ DCs in the MLN and in the inflamed ileal mucosa display reduced *Aldh1a2* expression compared with their steady-state counterparts (Collins et al., 2011). This correlates with reduced induction of Foxp3⁺ T_{REG} and enhanced induction of T_H17 cells, and again suggests that

a reduced ability of CD103⁺ DCs to produce RA may be involved in the pathogenesis of disease. As with the T cell transfer model, it is difficult to determine the extent to which these effects are the cause of intestinal inflammation or secondary to it. Overall, these reports indicate that CD103⁺ DCs may contribute to intestinal inflammation in mouse models of IBD through changes in the balance of effector T cell and T_{REG} induction.

1:15.2 Murine CD103⁻ DCs and Ly6C^{hi} monocyte-derived APCs in models of IBD

Both DSS and T cell transfer colitic mice display a significant increase in the proportion of CX₃CR1-GFP^{int} cells in the inflamed large intestinal mucosa of *Cx3cr1^{+/gfp}* mice (Rivollier et al., 2012, Bain et al., 2013, Tamoutounour et al., 2012, Zigmond et al., 2012, Weber et al., 2011a). This CX₃CR1-GFP^{int} population displays considerable heterogeneity, and the presence of conventional CD103⁻ DCs in this group is controversial. In T cell transfer colitis, a significant proportion of CX₃CR1-GFP^{int} cells are CD11c⁺ F4/80^{lo} CD103⁻ and E-cadherin⁺ and so are identified as CD103⁻ DCs. They express increased levels of IL-12, IL-23, TNF α and IL-6 compared with their steady-state CD103⁻ DC counterparts (Rivollier et al., 2012). CD103⁻ E-cadherin⁺ DCs simultaneously appear in inflamed intestinal mucosa and MLNs of T cell transfer colitis mice, but do not express CCR7. Despite an apparent lack of migration, they do however activate naive OT-II cells *in vitro* when extract from MLNs (Siddiqui et al., 2010).

The lack of migration into MLNs distinguishes CD103⁻ DCs in T cell transfer colitic mice from those in the steady state (Rivollier et al., 2012, Cerovic et al., 2012). One study identifies an influx of CX₃CR1-GFP^{int} cells simultaneously in the MLN and intestinal mucosa of T cell colitic mice and which stimulate naive OT-II cells, but instead identifies

them as 'inflammatory MΦ' (Tamoutounour et al., 2012). This identification is based on a MΦ morphology with vacuolar cytoplasm, contrasting with the non-vacuolar and dendritic morphology of migratory CD103⁻ DCs in the steady state (Cerovic et al., 2012).

Importantly, the CX₃CR1-GFP^{int} populations arriving in the inflamed intestinal mucosa of both T cell transfer and DSS colitic mice derive from circulating Ly6C^{hi} monocytes (Bain et al., 2013, Tamoutounour et al., 2012, Zigmond et al., 2012, Rivollier et al., 2012). Following differentiation within the mucosa, Ly6C^{hi} monocytes upregulate CD64 (FcγRI) expression, whilst conventional CD103⁺ and CD103⁻ DCs are CD11c⁺ CD64⁻. CD64 expression has therefore been proposed as a means of distinguishing conventional CD103⁻ DCs from the LyC^{hi} monocyte-derived CD103⁻ antigen presenting cells (APCs) which accumulate in inflammation (Tamoutounour et al., 2012). Furthermore, CD11c^{hi} CD103⁻ CD64⁻ cells in large intestinal mucosa expand in the presence of Flt3L in the steady state, consistent with a conventional DC phenotype (Bain et al., 2013).

In DSS colitic mice there is also an accumulation of Ly6C^{hi} monocyte-derived CX₃CR1-GFP^{int} cells in the inflamed intestinal mucosa (Zigmond et al., 2012, Bain et al., 2013). One study identifies increased lymphatic migration of CX₃CR1-GFP^{int} cells in DSS colitic mice (Zigmond et al., 2012). However it is not possible to determine whether this represents CD103⁻ Ly6C^{hi} monocyte-derived APCs which have become migratory in the presence of a DSS colitis-specific environment, or conventional CD103⁻ DCs which have known migratory capacity and are also CX₃CR1-GFP^{int}. A separate study using DSS colitis refers to the Ly6C^{hi} monocyte-derived CD64⁺ APCs as inflammatory MΦ, based on TNFα production in response to LPS and MΦ morphology (Bain et al., 2013). The considerable heterogeneity of the 'CD103⁻ DC' phenotype makes clear

characterisation of subsets difficult, whilst the disparate naming preferences of different groups further adds to the confusion.

The extent to which conventional CD103⁻ DCs and CD103⁻ Ly6C^{hi} monocyte-derived APCs contribute to pathogenesis in models of IBD is unclear. Both subsets induce IFN γ production following activation of naive OT-II cells (Rivollier et al., 2012, Tamoutounour et al., 2012). The importance of the arrival of Ly6C^{hi} monocytes into the intestinal mucosa in DSS colitis in the pathogenesis of disease is demonstrated in *CCR2*^{-/-} mice in which inflammation is significantly attenuated compared with WT mice (Platt et al., 2010). CCR2 expression by Ly6C^{hi} monocytes promotes both exit from the bone marrow (Serbina and Pamer, 2006) and entry into bacterially-infected peripheral tissues (Jia et al., 2008). Depletion of CCR2⁺ Ly6C^{hi} monocytes with a CCR2 monoclonal antibody also reduces the severity of DSS colitis (Zigmond et al., 2012).

Ly6C^{hi} monocytes entering acutely inflamed tissues including the spleen during bacterial infection differentiate into TNF/iNOS producing (Tip)-DCs (Serbina et al., 2003). In both DSS colitis and the adoptive T cell transfer model of colitis, CX₃CR1-GFP^{int} cells express iNOS (Weber et al., 2011a, Tamoutounour et al., 2012, Zigmond et al., 2012), indicating a possible overlap between the Ly6C^{hi}-monocyte derived APC and TipDC phenotype. Recruitment of Ly6C^{hi} monocytes through CCR2 signalling into the intestinal mucosa is required for the clearance of *Citrobacter rodentium* infection in mice (Kim et al., 2011). *Nod2*^{-/-} mice display reduced expression of the CCR2 ligand CCL2, leading to reduced recruitment of LyC^{hi} monocytes and reduced pathogen clearance (Kim et al., 2011). This may provide further explanation of the role of NOD2 mutations in susceptibility to CD as discussed in section 1:14.2.

Overall therefore, the arrival of Ly6C^{hi} monocytes and differentiation into CD103⁻ Ly6C^{hi} monocyte-derived APCs as opposed to the steady-state CX3CR1-GFP^{hi} resident MΦ phenotype is an important step in the pathogenesis of T cell transfer and DSS colitis. This phenotype may overlap with previous designations of 'CD103⁻ DCs' due to the lack of sufficient phenotypic detail. The human equivalent of the Ly6C^{hi} monocyte-derived DC population is the CD14⁺ monocyte-derived CD14⁺ MΦ and is discussed further in section 1:15.5.

1:15.3 Murine resident MΦ in models of IBD

CX3CR1-GFP^{hi} MΦ in the inflamed large intestinal mucosa of DSS-treated mice have a similar gene expression to that of their counterparts in non-treated healthy mice, indicating a resistance to changes within the inflammatory milieu (Zigmond et al., 2012). In the adoptive transfer model of colitis, CX3CR1-GFP^{hi} MΦ retain expression of IL-10, with a small increase in expression of pro-inflammatory IL-6, TNFα and iNOS compared with their counterparts in non-inflamed large intestine (Weber et al., 2011a). They also remain TREM-1⁻ and TLR2⁻ indicating low sensitivity to microbial stimulation even in the inflamed mucosa (Weber et al., 2011a). The absolute numbers of CX3CR1-GFP^{hi} MΦ increase in the large intestine of T cell transfer colitic mice, suggesting continued arrival and differentiation of Ly6C^{hi} monocytes or expansion of the local CX3CR1-GFP^{hi} MΦ population (Weber et al., 2011a).

1:15.4 Human intestinal DCs in IBD

Human intestinal DCs display significant phenotypic differences in IBD patients which may contribute to the pathogenesis of disease. When extracted from MLNs draining inflamed intestinal mucosa, DCs from CD patients induce a significantly greater level of

IFN γ production by CD4⁺ T cells compared with those derived from UC patients or from non-inflamed healthy controls (Sakuraba et al., 2009). The ability of intestinal DCs in CD patients to induce an enhanced T_H1 response is consistent with the increased production of IFN γ by LP T cells in CD (Fuss et al., 1996). The T_H1-favouring phenotype of MLN DCs in CD results from a combined increase in expression of IL-23 and reduced expression of IL-10, however IL-12 expression is unchanged compared with MLN DCs from healthy controls and UC patients (Sakuraba et al., 2009). In contrast, a significantly elevated proportion of DCs within human large intestinal mucosa contain intracellular IL-12 in CD compared with DCs in healthy controls and UC patients (Hart et al., 2005), which would promote induction of T_H1 cells.

Human DCs extracted from the large intestine of CD and UC patients express significantly increased levels of TLR2 and TLR4 compared with healthy controls. Furthermore, this expression is also increased in inflamed compared with paired non-inflamed mucosa in UC patients (Hart et al., 2005). This may indicate that DCs are more sensitive to stimulation by microbial products including LPS lipoproteins in IBD patients. Whether this reflects retention of TLR2 and TLR4 expression of blood DCs or *de novo* induction within the mucosa is unknown.

Expression of the co-stimulatory markers CD80, CD83 and CD86 are not significantly different on mDCs in healthy control and CD patients (Bell et al., 2001). CD40 expression is however significantly increased on mDCs in inflamed areas of CD patients compared with non-inflamed areas from the same patients and healthy controls (Hart et al., 2005). Furthermore, administration of anti-TNF α therapy to CD patients induced a significant decrease in CD40 expression on mDCs, irrespective of resolution of inflammation. This may suggest that TNF α in the intestinal mucosa of

active CD patients directly or indirectly upregulates CD40, but is insufficient to induce full maturation of mDCs.

One study has focussed on the CD1c⁺ human intestinal DC subset in IBD and confirms earlier observations of DCs. This includes an enhanced expression of TLR4 on these cells in both CD and UC patients compared with healthy controls, as well as increased production of TNF α and IL-8 following stimulation with LPS. They also display enhanced expression of CD40 which suggests a partially mature state (Baumgart et al., 2009). This study did not determine expression of CD103 on this subset; however CD1c⁺ DCs were predominantly CD103⁻ in a different study (Dillon et al., 2010). This suggests that they reflect CD103⁻ DC behaviour in CD and UC. Whether their ability to produce RA and induce a gut-homing or regulatory phenotype in IBD is modified is unknown. Human MLN CD103⁺ DCs in CD patients retain the ability to induce $\alpha_4\beta_7$ and CCR9 expression on naive CD8⁺ T cells (Jaensson et al., 2008), indicating that the production of RA by RALDH activity in human intestinal CD103⁺ DCs is conserved between healthy controls and CD patients. Whether production of RA by human CD103⁺ DCs is modified to the extent that other features, including the induction of Foxp3⁺ T_{REG}, are altered in CD whilst induction of imprinting is maintained is unclear.

1:15.5 Human intestinal M Φ in IBD

The inflamed intestinal mucosa of CD and UC patients contains a significantly increased frequency of CD14⁺ M Φ as a proportion of myeloid cells compared with healthy controls (Bain et al., 2013, Kamada et al., 2008, Tamoutounour et al., 2012, Grimm et al., 1995a). As with healthy controls, stimulation of the CD14⁺ M Φ population derived from the inflamed intestinal mucosa of CD and UC patients with heat-killed bacteria leads to the production of a range of pro-inflammatory cytokines including

TNF α and IL-6 (Kamada et al., 2008). In addition, stimulation of intestinal CD14⁺ M Φ from the inflamed mucosa of CD patients with heat-killed *E. faecalis* leads to the generation of significantly greater quantities of TNF α and IL-23 as compared with CD14⁺ M Φ from healthy controls or the inflamed intestinal mucosa of UC patients (Kamada et al., 2008). It has been proposed that the IL-23 produced by CD14⁺ M Φ in the inflamed intestinal mucosa of CD patients enhances IFN γ production by a subset of natural killer (NK) cells (Takayama et al., 2010), and that IL-23 also synergizes with TNF α to enhance production of IFN γ by CD4⁺ T cells in the intestinal mucosa (Kamada et al., 2008). Finally, CD14⁺ M Φ in the inflamed intestinal mucosa of CD patients express high levels of TNF-like factor (TL1A) (Kamada et al., 2010). TL1A acts as a co-stimulator molecule and induces secretion of pro-inflammatory cytokines by T cells (Migone et al., 2002). Synergy between IL-23 and TL1A expressed by CD14⁺ M Φ enhances induction of both IFN γ and IL-17 by intestinal mucosal T cells in CD patients (Kamada et al., 2010).

Human intestinal CD14⁺ M Φ share several phenotypic features with blood CD14⁺ monocytes including expression of the LPS co-receptor CD14 and the high affinity IgG receptor CD64 (Fc γ RI) (Kamada et al., 2008, Bain et al., 2013). Stimulation of CD14⁺ M Φ from the inflamed intestinal mucosa of CD and UC patients with immobilised IgG, which models IgG-immune complexes, through CD64 induces abundant TNF α and IL-1 β production as well as TL1A expression (Uo et al., 2012). In addition, both human CD14⁺ monocytes and CD14⁺ M Φ in inflamed intestinal mucosa of CD and UC patients express TREM-1 (Schenk et al., 2005, Schenk et al., 2007). Whilst the physiological ligand of TREM-1 is unknown, stimulation of intestinal CD14⁺ M Φ from CD and UC patients with TREM-1 monoclonal antibodies significantly increases production of a

range of pro-inflammatory cytokines including TNF α , IL-6 and IL-1 β (Schenk et al., 2007).

There is evidence of direct uptake of CD14⁺ monocytes into the inflamed intestinal mucosa of IBD patients (Grimm et al., 1995b). Furthermore, intestinal CD14⁺ M Φ are observed in close proximity to blood vessels within the inflamed intestinal mucosa of CD and UC patients (Rugtveit et al., 1994), indicating recent entry from circulation. Overall, this is consistent with the elevated proportion of CD14⁺ M Φ in inflamed intestinal mucosa of IBD patients compared with healthy controls as resulting from increased recruitment of CD14⁺ monocytes from circulation. As CD14⁺ blood monocytes have also been proposed as precursors to human intestinal resident M Φ (Smythies et al., 2005, Smythies et al., 2006), this suggests two distinct differentiation fates depending on the context in which the CD14⁺ monocyte arrives in the intestinal mucosa from circulation. In support of this model, the hyper IL-23-producing phenotype following stimulation with *E. faecalis* of CD14⁺ M Φ from the inflamed intestinal mucosa of CD patients can be generated by *in vitro* culture of CD14⁺ monocytes with M-CSF in the presence of IFN γ (Kamada et al., 2008).

M Φ from the inflamed large intestinal mucosa of CD and UC patients display elevated production of ROS as compared with healthy controls (Rugtveit et al., 1995). As circulating human monocytes produce ROS including hydrogen peroxide and superoxide (Nakagawara et al., 1981), this further supports the arrival of CD14⁺ monocytes into the intestinal mucosa in IBD. ROS produce by these cells may contribute to inflammation in the intestine by damaging the epithelium (reviewed by Nathan and Cunningham-Bussel, 2013)

CD14⁺ MΦ obtained from intestinal mucosa of healthy controls and CD patients are able to activate naive CD4⁺ T cells *in vitro* (Kamada et al., 2009). This is consistent with expression of the co-stimulatory markers CD40, CD80 and CD86 (Kamada et al., 2008). A significant proportion (> 30%) of proliferating CD4⁺ T cells activated *in vitro* by human intestinal CD14⁺ MΦ express IFNγ (T_H1) whilst a small proportion (< 0.5%) express IL-17A (T_H17). However, the absolute output of both IFNγ and IL-17A cytokine by CD4⁺ T cells activated by intestinal CD14⁺ MΦ from CD patients is significantly higher than T cells activated by intestinal CD14⁺ MΦ from healthy controls (Kamada et al., 2009). The increased ability of CD14⁺ MΦ from CD patients to induce IL-17A production by CD4⁺ T cells associates with reduced expression of the retinol dehydrogenase RDH10 (see section 1:12) in CD14⁺ MΦ, potentially resulting in reduced output of RA and so less suppression of IL-17A production (Kamada et al., 2009).

As human intestinal CD14⁺ MΦ do not express CCR7 (Kamada et al., 2008), they are unlikely to migrate into draining lymph nodes and interact with naive T cells. It is unclear whether they present antigen to local populations of effector T cells within the intestinal mucosa or simply influence cytokine production by these T cells through their own ability to produce cytokines (Kamada et al., 2008). Based on the assumption that CD14⁺ MΦ do present antigen to effector T cells, the increased proportion of CD14⁺ MΦ, particularly in CD, provides an increase range of targets for T_H1 cells to activate through the production of IFNγ. This would further support release of pro-inflammatory cytokines by CD14⁺ MΦ and contribute to the perpetuation of inflammation.

The behaviour of the human intestinal resident MΦ population (Smythies et al., 2005) within the inflamed mucosa of IBD patients is unknown. However, depletion of CD14⁺ cells from a mixed LP mononuclear cell (LPMC) preparation derived from the inflamed

intestinal mucosa of CD patients significantly reduces production of TNF α following stimulation with heat killed *E. faecalis* compared with stimulation of non-depleted LPMCs (Kamada et al., 2008). This indicates that the intestinal resident M Φ population within CD14⁺ cell-depleted LPMCs retains low or absent ability to produce pro-inflammatory cytokines upon microbial stimulation.

1:16 Lymphocyte gut-homing in the pathogenesis of IBD

1:16.1 $\alpha_4\beta_7$ -MAdCAM-1 and CCR9-CCL25 interactions in intestinal inflammation

Human CD103⁺ DCs obtained from MLNs draining inflamed TI of CD patients display an equivalent ability to induce $\alpha_4\beta_7$ and CCR9 as compared with those obtained from healthy control patients (Jaensson et al., 2008). This indicates that the level of RA output by human CD103⁺ DCs in this tissue during intestinal inflammation remains sufficient for the induction of these markers. Importantly, it also suggests that these pathways of lymphocyte migration remain active during intestinal inflammation.

Recruitment of CD4⁺ T cells into the colonic mucosa of mice in the adoptive T cell transfer model of colitis is significantly attenuated by administration of neutralising β_7 and MAdCAM-1 monoclonal antibodies (Picarella et al., 1997). This treatment also leads to significant histological improvement, underscoring the role of $\alpha_4\beta_7$ -MAdCAM-1 interactions in allowing entry of inflammatory CD4⁺ T cells into the intestinal mucosa in this model. In addition, migration of $\alpha_4\beta_7^+$ T and B cells into the distal colon of DSS-colitic mice is significantly attenuated by neutralising MAdCAM-1 antibodies (Kato et al., 2000). This correlates with reduced inflammation and improved colonic crypt architecture. In this model there is significant upregulation of MAdCAM-1 expression on

vascular endothelial cells in both the LP and sub-mucosa, indicating a key role during disease progression.

In humans, expression of MAdCAM-1 is also increased on vascular endothelial cells in inflamed intestinal mucosa in both UC and CD patients (Briskin et al., 1997, Souza et al., 1999). This suggests that activation of the endothelium during inflammation promotes $\alpha_4\beta_7$ -MAdCAM-1-mediated entry of lymphocytes into the human intestinal mucosa.

The proportion of $\alpha_4\beta_7^+$ T cells in the blood of CD and UC patients is reduced compared with healthy controls (Meenan et al., 1997, Hart et al., 2004). Potential explanations for this observation include increased uptake of $\alpha_4\beta_7^+$ T cells into the inflamed intestinal mucosa mediated by increased endothelial MAdCAM-1 expression, or reduced induction of $\alpha_4\beta_7$ during T cell activation.

Endothelial cells in the inflamed intestinal mucosa of IBD patients also express increased levels of ICAM-1 (Souza et al., 1999). This suggests that in addition to the steady-state pathways of lymphocyte migration into the gut, additional factors including expression of the ICAM-1-binding integrin $\alpha_L\beta_2$ (reviewed by Sigmundsdottir and Butcher, 2008) may mediate lymphocyte entry into the inflamed intestinal mucosa in humans. Migration of activated $CD4^+$ T cells into the ileal mucosa drives spontaneous inflammation in the SAMP/Fit mouse model of CD (Kosiewicz et al., 2001). In addition to $\alpha_4\beta_7$ -MAdCAM-1, this recruitment requires L-selectin and $\alpha_4\beta_1$ (Rivera-Nieves et al., 2005), which interacts with VCAM-1 on activated vascular endothelium (reviewed by Sigmundsdottir and Butcher, 2008). This further implicates a role for non-conventional gut-homing of lymphocytes during intestinal inflammation.

The frequency of CCR9⁺ T cells within the inflamed TI mucosa of CD patients is reduced compared with non-inflamed TI mucosa of these patients (Papadakis et al., 2001). The extent to which this is due to downregulation of CCR9 following entry into the tissue is difficult to determine, however it may reflect CCR9-CCL25-independent entry into of lymphocytes into the inflamed TI mucosa. This could be due to enhanced $\alpha_4\beta_7$ -MAdCAM-1 or $\alpha_L\beta_2$ -ICAM-1-mediated entry as described above. The reduced frequency of CCR9⁺ T cells in MLN draining inflamed TI of CD patients (Papadakis et al., 2001) may indicate reduced induction of CCR9 during T cell activation in MLN in these patients. *In vitro*, CD103⁺ DCs from MLN draining inflamed terminal ileum in CD patients induce similar levels of RA-dependent CCR9 on CD8 T⁺ cells compared with healthy control-derived cells (Jaensson et al., 2008). However there may be increased T cell activation by blood-derived CD103⁻ DC populations in these MLN that do not induce CCR9 (Jaensson et al., 2008). Patients with CD affecting the TI actually display increased proportions of CCR9⁺ T cells in peripheral blood compared with patients with colonic inflammation only (Papadakis et al., 2001). This suggests a counter argument that induction of CCR9 is actually increased in CD patients. Ultimately the key mechanisms of uptake and how this is regulated are not fully understood.

Gut-homing of T cells is likely to be key to the arrival both effector and regulatory T cells to the site of inflammation. However, there is evidence that in IBD lesions, effector T cells have resistance to TGF- β mediated regulation via expression of SMAD-7 (Fantini et al., 2009) Therefore the arrival of effector T cells is likely to be an important factor in driving the ongoing inflammatory episode.

1:16.2 Lymphocyte gut-homing in extra-intestinal manifestations in IBD

As discussed in section 1:14.1, up to 10 % of UC patients develop primary sclerosing cholangitis (PSC), where fibrosis causes inflammation and obstruction of the bile ducts (reviewed by Day and Morson, 2003). This may be due to aberrant expression of CCL25 and MAdCAM-1 on vascular endothelial cells leading to recruitment of $\alpha_4\beta_7^+$ CCR9⁺ T cells originally destined for the intestinal mucosa (Eksteen et al., 2004).

1:16.3 Gut-homing as a therapeutic target in IBD

The recruitment of lymphocytes into the intestinal mucosa in IBD has attracted considerable interest as a potential therapeutic target. This section will discuss the various targets and their respective stages of development, which is summarized in Figure 1.9)

Natalizumab is a humanised monoclonal antibody against α_4 integrin; it disrupts both $\alpha_4\beta_7$ -MAdCAM-1 as well as $\alpha_4\beta_1$ -VCAM-1 interactions. Clinical trials have demonstrated natalizumab performs significantly better than placebo at inducing remission in patients with moderate to severe CD (reviewed by MacDonald and McDonald, 2007). Patients on natalizumab also displayed significantly increased levels of circulating T and B cells consistent with impeded lymphocyte migration into peripheral tissues (Gordon et al., 2001). Compared with CD, much less is known about the efficacy of natalizumab in UC. An non placebo-controlled open label trial has however suggested that natalizumab may be of benefit in active UC (Gordon et al., 2002).

Safety concerns have been raised following cases of progressive multifocal leukoencephalopathy (PML) in CD and multiple sclerosis patients treated with natalizumab (Van Assche et al., 2005). In these cases interference with $\alpha_4\beta_1$ -VCAM-1

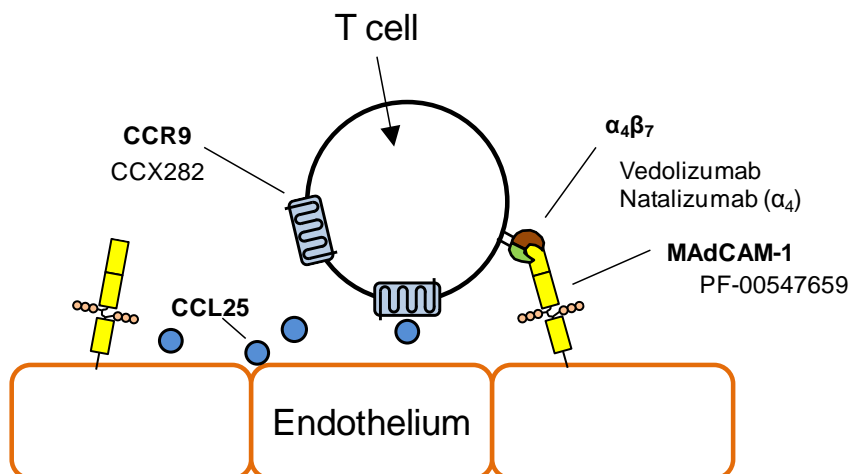


Figure 1.9: T cell gut-homing markers as therapeutic targets. Therapeutics in various stages of development include monoclonal antibodies to $\alpha_4\beta_7$ (Vedolizumab) and MAdCAM-1 (PF-00547659), as well as small molecule inhibitors of CCL25 (CCX282). Neutralising antibodies to α_4 are effective in CD but associated with rare fatalities including reactivation of latent JC virus in the central nervous system.

interactions may have impaired immunosurveillance of the central nervous system, leading to reactivation of latent John Cunningham (JC) virus. However, concomitant medication with other immunosuppressive drugs complicates the picture. Whilst this adverse reaction is relatively rare, it has caused fatalities. This may be avoided in future by screening patient plasma for presence of JC virus (MacDonald and McDonald, 2007).

Vedolizumab (MLN-02) is a humanized monoclonal antibody specific to $\alpha_4\beta_7$. In a phase II clinical trial it performed significantly better than placebo in inducing remission in active UC patients (Feagan et al., 2005). In a phase II trial, differences in clinical response for CD patients on vedolizumab were not significantly different from placebo (Feagan et al., 2008). Phase III trials for vedolizumab are ongoing for both CD and UC (Thomas and Baumgart, 2012). Vedolizumab has been shown to significantly reduce

the frequency of β_7^+ T cells in the intestinal mucosa of healthy cynomolgus monkeys, with a concurrent increase in peripheral blood β_7^+ T cells (Fedyk et al., 2012).

Current studies targeting MAdCAM-1 directly with a monoclonal antibody (PF-00547659) have shown some improvements upon endoscopic investigation in UC patients (Vermeire et al., 2009). This antibody is undergoing multiple trials to determine safety in humans (reviewed by Thomas and Baumgart, 2012).

CCX282 is an orally bio-available antagonist for CCR9, and has shown promise in reducing CD severity in a phase II trial (Eksteen and Adams, 2010). This approach is desirable as immune surveillance in the colon should remain unaffected. However, further trials are required before clear therapeutic benefit is demonstrated. The therapeutic targets based on lymphocyte homing to the intestinal mucosa are summarized in Figure 1.9.

1:17 RA in intestinal inflammation

RA was originally considered to be a universally pro-regulatory molecule in the murine intestine. This viewpoint derived from the involvement of RA in the induction of oral tolerance through enhancing the induction of Foxp3⁺ iT_{REG} in the murine MLN (Coombes et al., 2007, Sun et al., 2007, Mucida et al., 2007) (as discussed in section 1:8.5) and the loss of RALDH activity in CD103⁺ DCs in mouse models of intestinal inflammation (as discussed in section 1:15.1). However, RA signalling is also intimately involved in effector T cell responses which may contribute to inflammation. VAD mice display reduced clearance of the intracellular parasite *Toxoplasma gondii* following oral infection, which is mediated by a suppressed IFN γ ⁺ CD4⁺ T cell response (Hall et al., 2011a). A key component of this defective response is a failure of CD4⁺ T cells to

proliferate in response to the presentation of *T. gondii* antigen. This stems from the key role of RAR α in T cell activation through modulation of intracellular Ca²⁺ signalling (Hall et al., 2011a).

In addition to being necessary for effector T cell responses in the intestinal mucosa, there is also evidence that RA enhances pro-inflammatory responses under certain conditions. IL-15 promotes IL-12p70 production by murine intestinal DCs, and this production is further enhanced in the presence of RA (DePaolo et al., 2011). This is based on a synergistic phosphorylation of the MAPK JNK. The additional IL-12p70 suppresses induction of Foxp3⁺ T_{REG}, even in the presence of exogenous TGF- β . This has been proposed as a mechanism for the pathogenesis of Coeliac disease, where patients develop IFN γ ⁺ CD4⁺ T cell responses to the gluten-derived peptide gliadin (DePaolo et al., 2011).

There is currently an ongoing controversy as to whether isotretinoin (13-*cis* retinoic acid), prescribed for cystic acne, is causative in a small subset of IBD cases. However, population studies have found no significant association between isotretinoin use and IBD (Bernstein et al., 2009). This strongly suggests that this form of retinoic acid does not cause IBD, but cannot rule out that it may act as an IBD trigger in a small number of individuals.

The overall effects of RA on immune responses within steady state and inflamed intestinal mucosa may be determined by the particular profile of cytokines present, as well as the concentration of RA itself. In vitro, RA induces expression of $\alpha_4\beta_7$ and CCR9 on murine T cells from 0.1 nM (Iwata et al., 2004, Takeuchi et al., 2010). At 1 nM, RA promotes induction of T_H17 cells in the presence of IL-6 and TGF- β , (Takahashi et al., 2012), but does not support efficient induction of Foxp3⁺ T_{REG} in the presence of TGF- β

(Mucida et al., 2007, Takahashi et al., 2012). At 100 nM however, RA efficiently induces Foxp3⁺ T_{REG} in the presence of TGF- β whilst suppressing induction of T_H17 cells in the presence of IL-6 and TGF- β (Mucida et al., 2007, Takahashi et al., 2012). RA supports induction of IL-22 from $\gamma\delta$ T cells at 100 nM, but only in the presence of both IL-1 β and IL-23 (Mielke et al., 2013). These effects are summarised in Figure 1.10. Any potential role for RA in the pathogenesis of IBD in humans is therefore likely to result from a complex interplay of factors.

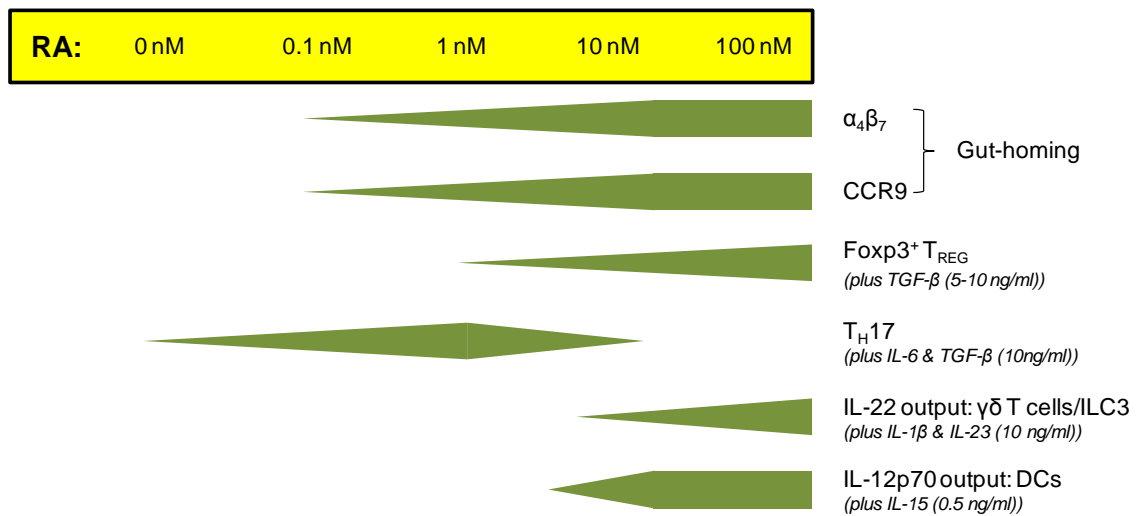


Figure 1.10: Concentration-dependence of RA effects in intestinal immunity. RA induces expression of gut-homing markers $\alpha_4\beta_7$ and CCR9 on T cells in mice from 0.1 nM. At 100 nM, RA enhances TGF- β mediated induction of Foxp3⁺ T_{REG} but suppresses induction of TGF- β and IL-6-mediated T_H17 induction *in vitro*. RA promotes T_H17 induction at 1 nM however, whilst at this concentration has no significant effect on induction of Foxp3⁺ T_{REG} *in vitro*. RA also enhances IL-22 production by innate lymphoid cells and $\gamma\delta$ T cells at 100 nM in the presence of IL-1 β and IL-23. In the presence of IL-15, 10 to 100 nM RA also promotes IL-12p70 production by murine DCs.

1:18 Hypothesis and aims

RA production by APCs, in particular CD103⁺ DCs, has emerged as an important contributor to immune regulation in the murine intestine. However, the role of RA production by APCs in the human intestine and whether this is involved in the pathogenesis of IBD is unknown. The overall hypothesis for this project is that the production of RA through RALDH enzyme activity by APCs including CD103⁺ DCs in the human intestinal mucosa is an important component of homeostasis in healthy individuals. Loss of this enzyme activity will associate with inflammation in the intestinal mucosa of IBD patients, and may contribute to the overall pathogenesis of disease. Restoration of this activity may therefore be an attractive therapeutic target.

The aims of this project will therefore be to validate a methodology for determining RALDH enzyme activity in APCs within human intestinal mucosa and then use this methodology to determine whether this activity is different in the healthy and inflamed human intestinal mucosa. The next aim will be to convert these observations into functional understanding of the ways in which these modifications may contribute to the pathogenesis of disease.

Chapter 2: Materials and methods

2:1 Reagents

Table 2.1: Reagents used in project

Reagent	Solvent	Stock conc. (mM)	Supplier
all- <i>trans</i> retinoic acid (RA)	DMSO	80	Sigma Aldrich
all- <i>trans</i> retinal	DMSO	80	Sigma Aldrich
AH6809	DMSO	16.8	Sigma Aldrich
Indomethacin	DMSO	1.68	Sigma Aldrich
Ro41-5253	DMSO	100	Enzo Life Sciences
CFSE	DMSO	40	Invitrogen

Reagents were stored at -80 °C; aliquots were discarded following the second freeze/thaw cycle to minimize degradation. Retinoids were stored in tubes covered with aluminium foil to minimize UV-induced degradation.

Complete medium: RPMI1640 Dutch Modification supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all Sigma-Aldrich) and 10% heat-treated (56 °C for 30 minutes) foetal calf serum (FCS; PAA).

FACS buffer: Phosphate-buffered saline solution (PBS) containing 2 %FCS, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2% (w/v) sodium azide.

MiniMACS buffer: PBS with BSA (0.5 %, w/v) and EDTA (2 mM)

2:2 Intestinal tissue and blood collection

2:2.1 Intestinal tissue

Up to 8 colonic and ileal biopsies were obtained at colonoscopy from patients with inflammatory bowel disease (IBD) or from control subjects, consisting of patients with macroscopically and histologically normal intestine who had been referred with symptoms of rectal bleeding or change in bowel habit with normal colonoscopic findings. Intestinal mucosa was also derived from surgically-resected tissue from IBD patients and from macroscopically unaffected areas of patients with colon cancer. The diagnosis for each patient was made using clinical parameters, radiographic studies and histological criteria. Tissue was collected in ice cold base medium (RPMI1640 Dutch Modification; Sigma-Aldrich).

Patient details are summarized in Appendix.

2:2.2 Blood

Blood was obtained by venepuncture from volunteer donors in 10 ml sodium heparin tubes (Becton Dickinson).

2:2.3 Ethics

All tissue and blood donors gave informed written consent and the study was approved by the local ethics committee (refs 05/Q0405/71, 08/H0702/33 and P/01/023).

2:3 Cell extraction from intestinal tissue

2:3.1 Lamina propria cells (LPCs)

Surgical resection specimens were cut into biopsy-sized pieces prior to tissue processing. LPCs were extracted from intestinal biopsies by enzymatic digestion as previously described (Bell et al., 2001, Hart et al., 2005). Biopsies were incubated for 20 minutes at room temperature with 1 mM DTT (Sigma-Aldrich) in calcium and magnesium-free Hank's balanced salt solution (HBSS; Sigma-Aldrich) in a T25 tissue culture flask (Becton Dickinson) to remove faeces and mucus. HBSS was removed from the flask with a Pasteur pipette and biopsies were washed in 10 ml additional HBSS which was then removed as previously. Biopsies were then incubated in 25 ml of 1 mM EDTA (Sigma-Aldrich) in HBSS at 37 °C under vigorous mechanical agitation for 30 minutes to remove the epithelium. Spent HBSS was removed followed by two washes and a further EDTA incubation as previously. Biopsies were incubated in 1 mg/ml collagenase D (Roche Applied Science) in pre-warmed HEPES buffered RPMI 1640 medium (Sigma-Aldrich) containing 2% heat inactivated (30 minutes at 56 °C) foetal calf serum (PAA) and 20 µg/ml deoxyribonuclease I (Roche Applied Science) in a fresh T25 flask at 37 °C under mechanical agitation. After 1 hour, the flask was shaken vigorously at 5 minute intervals until biopsies were fully digested. Cell suspension was passed through a 40 µm cell strainer (Becton Dickinson) and washed in base medium (RPMI1640).

2:3.2 'Walk out' cells

'Walk out' cells were derived from biopsies for use in T cell stimulation assays as described previously (Bell et al., 2001). Biopsies were treated with DTT and EDTA as

described in section 2:3.1, then cultured overnight at 37 °C in 1 ml complete medium (RPMI1640 Dutch Modification supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all Sigma-Aldrich) and 10% foetal calf serum (PAA)) in 24 well plates (Becton Dickinson) at one biopsy per well.

APC-enriched cells present in the culture medium were recovered by centrifugation following removal of residual biopsy tissue.

2:3.3 Flow-sorted APC populations

LPCs were washed into filter sterilised modified FACS buffer (lacking sodium azide) and incubated with relevant monoclonal antibodies (see Table 2.1). APC populations within LPCs were isolated by flow-sorting with a FACS Aria (Becton Dickinson). DCs were isolated as CD45⁺ Lin⁻ HLA-DR⁺ cells, and CD14⁺ MΦ were isolated as CD33⁺ CD14⁺ cells. The discrete nature of antibody staining for these populations allowed for cell identification without the need for isotype controls. Cells were sorted at the lowest speed possible to minimise sort-associated cell damage, and were collected in sterile 1.5 ml TubeOne Microcentrifuge Tubes (Star Lab) containing 0.5 ml complete medium. Technical assistance for cell sorting was provided by Gary Warnes, Flow Cytometry Core Facility, Blizzard Institute.

2:4 Purification of blood cell populations

2:4.1 Peripheral blood mononuclear cells (PBMCs)

Peripheral blood was diluted 2:1 with RPMI1640 Dutch Modification base medium, then layered over 15 ml Ficoll-Paque PLUS (GE Healthcare) in 15 ml falcon tubes using a Pasteur pipette and centrifuged at 650 x g for 20 minutes with the brake at its lowest

setting or deactivated. PBMCs were collected from the interface between Ficoll and serum with a Pasteur pipette, pooled into 15 ml Falcon tubes (Becton Dickinson) and centrifuged at 650 x g for 10 minutes. Supernatant was discarded and pellets were resuspended in residual volume, pooled into a 5 ml FACS tube (Becton Dickinson) and washed twice into complete medium by centrifugation at 400 x g for 5 minutes.

2:4.2 Naive CD4⁺ T cells

PBMCs were washed into sterile filtered miniMACS buffer and resuspended in 40 µl miniMACS buffer for every 10 ml blood originally used. Naive CD4⁺ T cells were then purified by negative selection using Human Naive CD4⁺ T Cell kit (Miltenyi Biotec). Briefly, PBMCs were incubated with 10 µl antibody cocktail II per 10 ml of blood for 15 minutes on ice. This cocktail contains antibodies targeting antigens associated with all haematopoietic cell lineages except CD4⁺ T cells including CD8, CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCRγ/δ, HLA-DR and CD235a. Effector/memory CD4⁺ T cells are excluded by antibodies to CD45RO and CD25. After labelling with the antibody cocktail, cells were washed by adding 2 ml of miniMACS buffer followed by centrifugation at 400 x g for 5 minutes. Cell pellet was resuspended in 80 µl miniMACS buffer per 10 ml blood and then incubated with 20 µl anti-biotin microbeads per 10 ml of blood for a further 10 minutes on ice. Cells were then washed by adding 2 ml of miniMACS buffer followed by centrifugation at 400 x g for 5 minutes resuspended in 500 µl miniMACS buffer. An LS column was used for the separation to allow for the high level of non-target cell binding in negative selection. The column was also pre-chilled to increase the stability of antibody binding. The LS column was attached to the magnet and separation apparatus and 3 ml miniMACS buffer added and allowed to run through the column completely. The labelled PBMC suspension was then added to the

column, allowed to run in and washed through the column by 3 sequential additions of 3 ml miniMACS buffer. The eluted fraction enriched for naive CD4⁺ T cells was collected and was enriched for naive CD4⁺ T cells. Purity was verified by flow cytometry and was routinely >96 %. Cells magnetically attached the column were released by adding a further 5 ml miniMACS buffer with the column removed from the magnet, and depressing the plunger. This fraction contained PBMCs depleted of naive CD4⁺ T cells and was used for compensation control staining in flow cytometry (see below).

2:4.3 CD14⁺ monocytes

PBMCs were centrifuged at 400 x g for 5 minutes, supernatant discarded and resuspended in 2 ml miniMACS buffer before being centrifuged again at 400 x g for 5 minutes. Cell pellet was resuspended in 100 µl miniMACS buffer, labelled with 10 µl anti-CD14 microbeads (Miltenyi Biotec) per 10 ml of blood and incubated for 15 minutes on ice. An MS column was used for the purification of CD14⁺ monocytes as the number of labelled cells was not great enough to warrant use of the greater capacity LS column. The MS column was also pre-chilled to increase the stability of antibody binding, and assembled with the appropriate magnet onto the separation apparatus. MiniMACS buffer (0.5 ml) was added to the column and allowed to run through completely before the labelled cell suspension was added. This was followed by 3 sequential additions of 500 µl miniMACS buffer. Eluted cells contained PBMCs depleted of CD14⁺ monocytes. Cells magnetically attached to the column were eluted by removing the column from the magnet and adding a further 500 µl miniMACS buffer and depressing the plunger. This fraction was enriched for CD14⁺ monocytes; purity was verified by flow cytometry and was routinely >98 %. Monocytes were washed twice into complete medium by centrifugation at 400 x g for 5 minutes.

2:4.4 Cell counts

Cell counts were performed by mixing 50 µl cell suspension with 50 µl Trypan Blue Solution (0.4%, Invitrogen) and 150 µl RPMI-Dutch modification. A small sample of this mixture was applied to a haemocytometer (Neubauer) and cells counted within a 4 x 4 grid. An average count was taken from at least 2 grids and this number was converted to the cell concentration by the following formula:

$$\begin{array}{c} \text{Average number of cells} \\ \text{in each 4 x 4 grid} \end{array} \times 5 \times 10^4 = \text{cells/ml}$$

2:5 *In vitro* cell culture

2:5.1 Monocyte-derived DCs (MoDCs)

CD14⁺ monocytes were obtained from PBMCs as described above. They were then cultured in the presence of GM-CSF (100 ng/ml) and IL-4 (100 ng/ml; both Peprotech) at 5 x 10⁵ cells/ml in 1 ml of complete medium in 24 well plates at 37 °C, 5 % CO₂ in a humidified incubator. Half of the spent culture medium was removed on day 4 and replaced with fresh medium, GM-CSF and IL-4 to the same final concentration of 100 ng/ml. Cells were collected for functional analysis on day 7.

In experiments where MoDCs were conditioned with intestinal biopsy conditioned medium (CM), the final culture volume was reduced to 600 µl and CM introduced from the beginning of culture at 20% (v/v). The culture volume was reduced so as to conserve the CM samples. The total number of CD14⁺ monocytes was kept at 5 x 10⁵ whilst the concentration of GM-CSF and IL-4 was maintained (100 ng/ml).

Intestinal biopsy conditioned medium (CM) was generated with DTT-treated intestinal biopsies (as described in section 2:3.1) by culturing one biopsy per well in a 24 well plate in 1 ml of complete medium for 6 hours in a humidified incubator at 37 °C with 5 % CO₂. Biopsies were then removed and CM pooled for each condition, filter sterilized and stored at -80 °C.

Maturation of MoDCs was induced *in vitro* in some experiments by culturing MoDCs (1×10^5) in the presence or absence of LPS (1 µg/ml) in 0.5 ml complete medium overnight in a humidified incubator at 37 °C with 5% CO₂.

2:5.2 Inflammatory CD14⁺ MΦ

CD14⁺ monocytes were purified from PBMCs as described above. They were then cultured in the presence of GM-CSF (100 ng/ml) at 5×10^5 cells/ml in 1 ml complete medium in 24 well plates (Becton Dickinson) at 37 °C, 5 % CO₂ in a humidified incubator. Half of the spent culture medium was removed on day 3 and replaced with fresh medium and GM-CSF (to a final concentration of 100 ng/ml). CD14⁺ MΦ were then cultured on day 6 at 1×10^5 cells/ml in the presence or absence of LPS (1 µg/ml, *Escherichia coli* 0111:B4, Sigma-Aldrich) in 1 ml complete medium in the presence of Brefeldin A (10 µg/ml, Sigma-Alrich). Cells were then prepared for intracellular cytokine staining and analysis by flow cytometry as described below.

2:5.3 Activation of naïve CD4⁺ T cells

2:5.3.1 CFSE labelling

Naïve CD4⁺ T cells were purified from PBMCs as described above. They were then resuspended in serum-free PBS filter sterilised with a 0.2 µm syringe filter (VWR) at

approximately 2×10^7 cells/ml and stained with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) at a final concentration of $5 \mu\text{M}$ for 3 minutes at room temperature. Labelling was quenched the addition of one volume of FCS and then cells were washed twice by centrifugation ($400 \times g$, 5 minutes) in complete medium.

2:5.3.2 *Activation of allogeneic naive CD4⁺ T cells*

CFSE-labelled allogeneic naive CD4⁺ T cells (2×10^5) were cultured with either 5×10^4 'walk out' cells or alternatively 2×10^4 sorted intestinal APCs or MoDCs for 5 days in $200 \mu\text{l}$ complete medium in round bottom 96 well plates (Becton Dickinson) at 37°C , 5 % CO_2 in a humidified incubator. Cells were recovered on day 5 or 6 and proliferating CFSE^{lo} cells identified and analysed by flow cytometry as described below.

2:5.3.3 *Activation of naive CD4⁺ T cells with activation beads*

In some experiments naive CD4⁺ T cells were activated with activation beads (Miltenyi Biotec). Activation beads were prepared according to manufacturer's instructions. In brief, $62.5 \mu\text{l}$ of anti-biotin MACSi beads were mixed with $12.5 \mu\text{l}$ each of biotinylated anti-CD2, CD3 and CD28 and $25 \mu\text{l}$ of miniMACS buffer. This suspension was incubated under mechanical rotation for 2 hours at 4°C . A sample ($25 \mu\text{l}$) of this suspension was then transferred to a 1.5 ml sterile screw cap tube (Star Lab) with the addition of $200 \mu\text{l}$ of complete medium. Following centrifugation ($400 \times g$, 5 minutes), the supernatant was removed by pipetting and the bead pellet resuspended in $100 \mu\text{l}$ complete medium. CFSE-labelled naive CD4⁺ T cells (2×10^5) were cultured in the presence of $5 \mu\text{l}$ of this activation bead suspension in complete medium at a final volume of $200 \mu\text{l}$ and analysed by flow cytometry on day 5 or 6 as described below.

2:6 Aldefluor assay

Flow cytometric analysis of cellular RALDH activity was performed with the Aldefluor assay (Stemcell Technologies). The reaction mechanism is described in detail in Chapter 3 (section 3:2). LPCs (4×10^6) and PBMCs or M Φ (1×10^6) were washed in PBS by centrifugation (400 x g, 5 minutes) and resuspended in 1 ml Aldefluor assay buffer in a 5 ml tube labelled 'test'. Elevated numbers of LPCs were used to facilitate identification of RALDH activity in rare DC populations. 5 μ l of the ALDH inhibitor diethylaminobenzaldehyde (DEAB) was placed into a second 5 ml tube labelled 'control'. Aldefluor reagent (5 μ l) was then added to 'test' (final concentration 1.5 μ M); the cells were mixed and half the sample (500 μ l) was transferred and mixed in the 'control' tube. The tubes were then incubated at 37°C for 45 minutes wrapped in aluminium foil, then prepared for flow cytometry (see below). Aldefluor^{hi} cells were determined during data analysis by gating for cells at a fixed separation (2 cm on screen in all samples) from the leading edge of the DEAB-inhibited 'control' sample. Each cell subpopulation was defined in this way relative to its specific DEAB-inhibited 'control' sample.

2:7 Flow cytometry

2:7.1 Antibodies

Table 2.2: Antibodies used for flow cytometry

Antigen	Fluorochrome	Clone	Isotype	Supplier
CD33	APC	WM53	Mouse IgG1	Biolegend
CD11c	APC	3.9	Mouse IgG1	Biolegend
CD4	APC	RPA-T4	Mouse IgG1	Biolegend
CD45	Pacific blue	HI30	Mouse IgG1	Biolegend
CD14	Pacific blue	M5E2	Mouse IgG2a	Biolegend

CCR7	Pacific blue	G043H7	Mouse IgG2a	Biolegend
CD103	PE	LF61	Mouse IgG1	Biolegend
CD70	PE	113-16	Mouse IgG1	Biolegend
E-Cadherin	PE	67A4	Mouse IgG1	Biolegend
Beta7	PE	FIB504	Rat IgG2a	Biolegend
CD19	PE	HIB19	Mouse IgG1	Biolegend
TNF α	PE	MAb11	Mouse IgG1	Biolegend
Goat anti-mouse IgG	PE	Poly4053		Biolegend
HLA-DR	PE/Cy7	L243	Mouse IgG2a	Biolegend
CD209	FITC	9E9A8	Mouse IgG2a	Biolegend
Lineage*	PE/Cy5			AbD Serotec
CX ₃ CR1	FITC	FAB5204F	Mouse IgG1	R&D Systems
CD8	PerCP/Cy5.5	SK-1	Mouse IgG1	BD Biosciences
pSTAT5	Alexafluor 647	47/Stat5(pY694)	Mouse IgG1	BD Biosciences

* Lineage cocktail: anti-CD3, CD14, CD16, CD19 and CD34

Isotype-matched control antibodies were obtained from the appropriate manufacturer.

Act-1 (anti-human $\alpha_4\beta_7$) was a kind gift from Eugene Butcher, Stanford University.

LP34 (anti human cytokeratin 8/16) was a kind gift from Kristin Braun, Barts and the London School of Medicine and Dentistry.

2:7.2 Antibody labelling

Cells were transferred into 5 ml FACS tubes (Becton Dickinson) and washed by centrifugation (400 x g, 5 minutes) in cold FACS buffer. Supernatant was removed by inversion, and monoclonal antibodies were added to the residual volume (approximately 100 μ l) containing the cell pellet at 5 μ l antibody per test. Cells were vortexed and incubated on ice for 15 minutes and washed twice by centrifugation (400 x g, 5 minutes) and supernatant removed by inversion.

For detection of intracellular antigen e.g. cytokines and cytokeratins, cells were fixed following extracellular labelling by addition of 100 μ l Leucoperm A solution (AbD

Serotec) and vortexed, then incubated in the dark for 15 minutes at room temperature. Cells were then washed by the centrifugation (400 x g, 5 minutes) with ice cold FACS buffer, supernatant removed entirely by inversion of the tube onto paper towel to remove residual volume. Cells were permeabilised with 100 μ l Leucoperm B solution (AbD Serotec) in the presence of relevant monoclonal antibodies (5 μ l/test), vortexed and incubated at room temperature in the dark for 30 minutes. Cells were then washed in cold FACS buffer by centrifugation (400 x g, 5 minutes) and fixed in paraformaldehyde (1 % w/v).

Viability was assessed in some unfixed cell populations by the addition of 3 μ l 7-amino-actinomycin D (7-AAD; Biolegend) in 300 μ l FACS buffer. Samples were vortexed, incubated for 5 minutes at room temperature and then analysed by flow cytometry (see below). 7-AAD enters cells with a damaged cell membrane (i.e. dead cells) and complexes with DNA, but is restricted to the exterior of cells with a viable membrane (i.e. live cells), providing a clear distinction between live and dead cells upon data analysis.

2:7.3 Flow cytometry

Labelled cells were acquired on a Canto II or LSR II (Becton Dickinson) using CellQuest software (Becton Dickinson). Single fluorophore compensation controls were generated by labelling compensation beads (Becton Dickinson) with the individual antibodies used in a particular experiment. These contain a population of beads that non-specifically binds to mouse IgG, generating a positively stained population, and beads to do not bind to mouse IgG and so create a negatively stained population. In cases where rat IgG was used (e.g. staining for β_7), PBMCs labelled with only this antibody were used as the compensation control. In the case of 7-AAD staining, 1×10^6

PBMCs were cultured at 60 °C in PBS for 5 minutes and then placed on ice, and then combined with 1×10^6 PBMCs in PBD at room temperature. The combined cell population contained equal amounts of live and dead cells, and so when stained with 7-AAD immediately prior to flow cytometry provides a single fluorophore compensation control for 7-AAD.

At least 10,000 events were acquired for each sample, except in the analysis of Aldefluor activity in LPCs in which up to 300,000 events were acquired due to the rarity of DC populations within these samples. Data were exported and analysed using WinList 6.0 software (Verity). Compensation was applied prior to data analysis using the single fluorophore controls described above. Positive staining was determined by comparison with populations within samples stained with matched isotype control antibodies. Alternatively, for some populations this distinction was made on the basis of discrete staining characteristics e.g. CD45, where there is a clear distinction between positive and negatively stained populations. The level of staining was quantified as the mean fluorescence intensity (MFI), and geometric mean was used throughout so that the mean values were not distorted by the high levels of fluorescence of positively stained populations.

2:7.4 PhosFlow analysis of STAT5 in MoDCs

CD14⁺ monocytes were cultured in the presence of GM-CSF and IL-4 (100 ng/ml) in complete medium in the presence or absence of intestinal biopsy-derived CM. Phosphorylation of STAT5 was determined using a methodology developed in house. Briefly, cells were fixed in PFA (4 %) following 0, 0.25, 1, 4 or 24 hours of culture and permeabilised in ice cold methanol (70 %), labelled with monoclonal anti-phospho-STAT5 antibody and analysed by flow cytometry.

2:8 Quantitative real-time PCR

2:8.1 RNA extraction

Cells were washed in PBS by centrifugation (400 x g, 5 minutes), then supernatant was removed entirely by careful pipetting. RNA extraction was performed using the RNeasy Micro Kit (Qiagen) as per manufacturer's instruction. In brief, samples with low numbers of cells ($<1 \times 10^5$) were lysed with 75 μ l RLT buffer by vortexing for 1 minute. Alternatively, samples with ($>1 \times 10^5$ cells) were lysed in 350 μ l RLT buffer by vortexing for 1 minute. RNA extraction was either performed immediately after this point, or alternatively the cell lysate was stored at -80 °C and thawed at a later date in a water bath at 37 °C before continuing RNA extraction. One volume of 70 % ethanol (i.e. either 75 or 350 μ l) was added to the cell lysate and mixed by repeated pipetting. The entire volume was then transferred onto an RNeasy MinElute spin column placed in a 2 ml collection tube and centrifuged (8,000 x g, 15 seconds). Flow-through liquid in the collection tube was discarded and the collection tube re-used. RNA trapped on the column membrane was washed by the addition of 700 μ l RWI buffer, and centrifugation (8,000 x g, 15 seconds). The RNeasy MinElute spin column was then placed in a fresh 2 ml collection tube, then 500 μ l RPE buffer was added followed by centrifugation (8,000 x g, 15 seconds) to wash the spin column membrane. The flow-through liquid was then discarded and 2 ml collection tube re-used. 500 μ l of 80 % ethanol was then added to the column to wash the membrane, followed by centrifugation (8,000 x g, 2 minutes). The spin column was then placed in a fresh 2 ml collection tube and centrifuged at full speed for 5 minutes with the lid of the spin column open to completely remove all ethanol from the membrane. Finally, the spin column was transferred to a 1.5 ml collection tube, 14 μ l of RNase-free water was added directly to the membrane and then spin column was centrifuged at full speed for 1 minute to elute the RNA.

Purified RNA was used either immediately for reverse transcription to cDNA (see below) or stored at -20 °C for processing at a later date.

2:8.2 Reverse transcription

Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen) as per manufacturer's instruction. In brief, the entire RNA sample (approximately 12 µl) was incubated with 2 µl gDNA Wipeout Buffer for 2 minutes at 42 °C to degrade genomic DNA. The 14 µl gDNA elimination reaction was then mixed with Quantiscript Reverse Transcriptase (1 µl), Quantitect RT Buffer (4 µl) and RT primer mix (1 µl) and incubated at 42 °C for minutes allowing reverse transcription of all RNA molecules present into cDNA. The sample was then incubated at 95 °C for 3 minutes to inactivate Quantiscript Reverse Transcriptase. The cDNA sample was then either used for real-time PCR or stored at -20 °C until required.

2:8.3 Real-time PCR with SYBR Green

Quantitative real-time PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen) as per manufacturer's instruction. In brief, master mixes containing 2x QuantiFast SYBR Green PCR Master Mix (12.5 µl), specific forward and reverse primers (primers listed in Table 2.3; 2.5 µl each or 2.5 µl of custom primers in which forward and reverse primers are combined for a final concentration of 1 µM), and sufficient RNase-free water to bring the volume up to 24 µl for each amplification. This master mix was then pipetted into the wells of a 96 well PCR plate (24 µl/well; Star Lab). cDNA (1 µl) was added to the top of relevant wells, then the plate was covered with Advanced Polyolefin StarSeal (Star Lab) and briefly centrifuged to draw the cDNA droplet into the master mix at the base of the well. The plate was then run on a 7500

Real-Time PCR System (Applied Biosystems). The program used for all amplifications is displayed in Table 2.4.

Table 2.3: Primers used in real-time PCR

Target	Forward primer	Reverse primer	Company	Cat. number
<i>ALDH1A1</i>	GTTGATCAAAGAAGCTGCCG	CAACAGCATTGTCCAAGTCG	Invitrogen	N/A
<i>ALDH1A2</i>	CACTGATGATATGCGGATTGC	TAAAGACAGCTGCTACGAGTC	Invitrogen	N/A
<i>ALDH1A3</i>	GAATAGCACCGACTATGGAC	AGCAGTTGATCCAGACCGTT	Invitrogen	N/A
<i>DHRS9</i>	TCAGAGAGACTTCGTACTGTG	TGTAGTCCTCTAGTGTGAGCC	Invitrogen	N/A
<i>GAPDH</i>	TGCACCACCAACTGCTTAGC	GCATGGACTGTGGTCATGAG	Invitrogen	N/A
<i>RDH10</i>			Qiagen	QT00029176
<i>RDH16</i>			Qiagen	QT00493024
<i>MN1</i>			Qiagen	QT00043414
<i>RPL30</i>			Qiagen	QT00056651

Table 2.4: PCR program

Step	Time	Temperature
1. Activation step	5 min	95 °C
2. Denaturation	10 sec	95 °C
3. Annealing / extension	33 sec	60 °C

Data was collected at step 3, and steps 2 to 3 were cycled 40 times. Following amplification, a threshold was placed across the beginning of the log-linear phase of amplification and the cycle number at which the individual amplifications crossed this threshold (Ct value) were collected. Expression of the target gene (e.g. *ALDH1A1*) was normalized to the reference genes *GAPDH* or *RPL30* using the $2^{-\Delta C_t}$ method (Pfaffl, 2001) where ΔC_t is the difference in threshold cycle number for target and reference gene. *RPL30* is a newly identified reference gene encoding a ribosomal protein with exceptionally stable expression across a wide range of human tissues and following different drug treatments (de Jonge et al., 2007). Where indicated in the text, a

geometric mean value of expression relative to both *GAPDH* and *RPL30* is reported. An example of an amplification plot (*ALDH1A1*) is displayed in Figure 2.1A. All amplifications were run in the presence of a no template control (NTC) containing water instead of cDNA to ensure that any amplification was not the result of contamination of the reagents.

Purity of amplified product and the presence of primer dimers were determined by running a melt curve analysis with every experiment. This step involves incremental increases in temperature and analysis of fluorescence at each increase. SYBR green is fluorescent in the presence of double stranded DNA, and the dissociation of double stranded DNA therefore leads to a loss of this fluorescence. The temperature at which double stranded DNA dissociates or 'melts' results from the size of the molecule, and the composition of GC and AT base pairs. If the amplified PCR product is pure, all the DNA molecules will melt at the same temperature, leading to a large loss of fluorescence at a specific temperature. However, where multiple products have been non-specifically generated, SYBR green fluorescence is lost at a range of temperatures associated with the distinct melting points of these various products. Primer dimers also produce a characteristic melt curve profile with a lower melting temperature than the desired product. Therefore, if amplification is detected but only primer dimers are observed by melt curve analysis, this may be taken as a negative result. An example of melt curve analysis for *ALDH1A1* amplification is displayed in Figure 2.1B.

Primers used were either custom made from Qiagen, or were designed in house. In all cases, primers were exon spanning so as to prevent amplification of contaminating genomic DNA versions of the target gene.

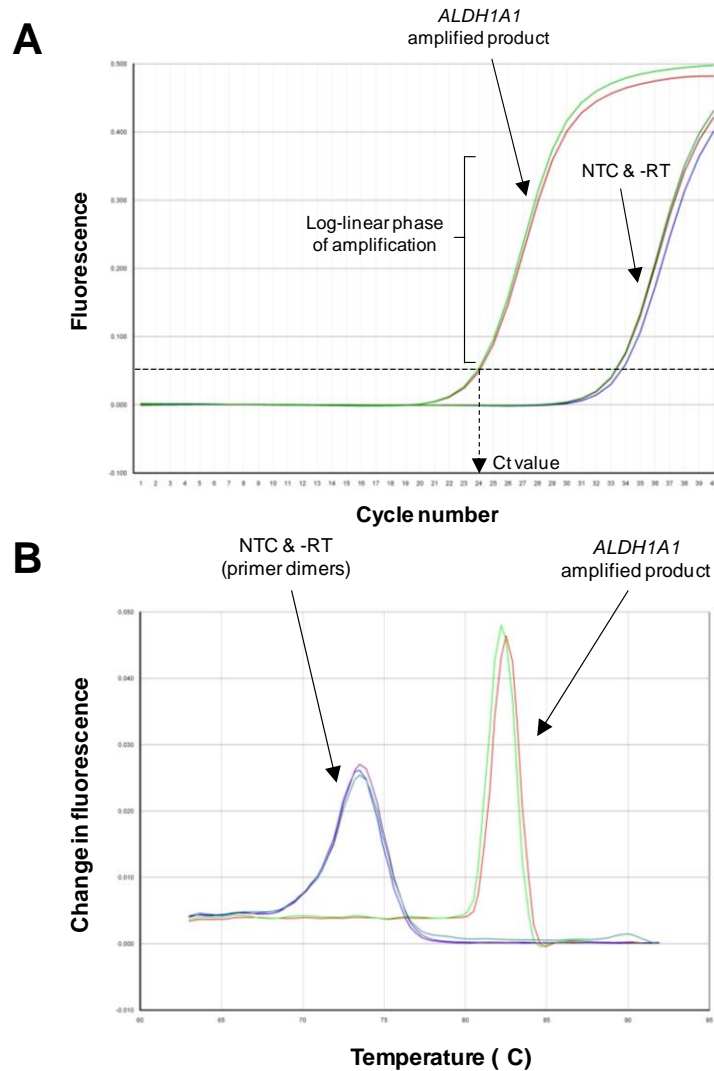


Figure 2.1: Example of real time PCR amplification. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 6 days, then RNA was extracted and reverse transcribed to cDNA. Real time PCR with SYBR green was performed using primers specific for *ALDH1A1*. Amplification was also performed with *ALDH1A1* primers either in absence of cDNA (no template control; NTC) or with a sample in which reverse transcriptase was omitted from the reverse transcription step (i.e. no cDNA present; -RT). A threshold was placed at the beginning of the log-linear phase of amplification, with the cycle number at which the fluorescence crosses this line as the Ct value. **B:** Purity of the amplified produce was verified by melt curve analysis. For the *ALDH1A1* amplified product there is a single discrete change in fluorescence at a specific temperature indicating a pure product. The NTC and -RT controls display the classic melt curve profile of primer dimers, indicating that the low level amplification observed for these samples in A is the result of primer dimer formation.

2:9 Analysis of retinoid concentrations

2:9.1 MoDC cultures

MoDCs were differentiated from CD14⁺ monocytes as described in Section 2:2.1, in the presence or absence of intestinal biopsy CM. Cells were washed twice by centrifugation in complete medium (400 x g, 5 minutes) and cultured at 5×10^5 cells/well in a 24 well plate for 4 hours in the presence or absence of *all-trans* retinal (100 nM; Sigma-Aldrich). Cells were then collected and centrifuged (400 x g, 5 minutes), supernatant was removed and then both supernatant and cell pellet were snap frozen in liquid nitrogen for analysis of retinoid concentration as described below.

2:9.2 Intestinal biopsies

Intestinal biopsies were collected from patients in absence of RPMI and snap frozen in liquid nitrogen as soon as possible to limit degradation of retinoids. Retinoid concentration was determined as described below.

2:9.3 Retinoid quantification

Retinoic acid, retinol and retinyl esters were quantified by high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) in the laboratory of Maureen Kane, University of Maryland, Baltimore, as described previously (Kane et al., 2005).

2:10 Statistics

Statistical analyses were performed using Sigmaplot 11.0 (Systat Software, London, UK). Comparisons between two groups of normally distributed data were analysed

using t-tests, paired where appropriate. Datasets containing more than two groups were compared by analysis of variance (ANOVA) with pair-wise comparisons using the Tukey test to correct for multiple comparisons. Non-normally distributed data were \log_{10} transformed and then analysed by ANOVA and Tukey test where indicated in the text. P-values were regarded as statistically significant when $p < 0.05$. Significance of correlations for non-normally distributed data assessed by Spearman's Rank.

Chapter 3: Validation of Aldefluor assay for determining RALDH activity in human dendritic cells

3:1 Chapter summary

This Chapter focuses on determining the suitability of the Aldefluor assay for assessing RALDH activity in human DCs by exploring the regulation of Aldefluor staining and RALDH gene expression in MoDCs as a model. MoDCs demonstrate Aldefluor activity with elevated expression of multiple *ALDH1A* isoforms in Aldefluor^{hi} cells. This activity is also competitively inhibited by retinal, indicating a close association between Aldefluor and RALDH activity. Consistent with our understanding of the regulation of RALDH activity in other cells, RAR α signalling during differentiation of MoDCs is required for *ALDH1A* expression and Aldefluor/RALDH activity, whilst exogenous RA enhances Aldefluor/RALDH activity through increased *ALDH1A2* expression. Interestingly, Aldefluor/RALDH is greatest in MoDCs with a mature phenotype and consequently may be enhanced following LPS stimulation as the frequency of mature MoDCs increases. GM-CSF alone is a potent inducer of Aldefluor/RALDH activity in human monocytes, whereas IL-4 appears to act as a negative RALDH regulator which attenuates GM-CSF-mediated induction. In conclusion, the Aldefluor assay is suitable for analysis of RALDH activity in human MoDCs, and is therefore a valid approach for the analysis of RA generation in human intestinal DCs.

3:2 Introduction

The production of RA through RALDH enzyme activity is critical for the ability of murine small intestinal and MLN CD103⁺ DCs to imprint a gut-homing $\alpha_4\beta_7^+$ and CCR9⁺

phenotype during T cell activation (Iwata et al., 2004 and reviewed by Agace, 2010). RA also supports the TGF- β mediated induction of Foxp3⁺ T_{REG} from naive CD4⁺ T cells and suppresses induction of T_H17 cells (Mucida et al., 2007, Sun et al., 2007, Coombes et al., 2007, Schambach et al., 2007). The loss of RALDH activity by intestinal CD103⁺ DCs is implicated in the pathogenesis of experimental intestinal inflammation of mice (Collins et al., 2011, Laffont et al., 2010). It has been demonstrated that human CD103⁺ but not CD103⁻ MLN DCs induce high levels of CCR9 expression on CD8⁺ T cells, consistent with an enhanced ability to generate RA via the activity of RALDH enzymes (Jaensson et al., 2008). However, the role of RALDH activity in human intestinal DCs and whether modification of this activity plays a role in IBD remains poorly defined.

RALDH activity in murine intestinal CD103⁺ DCs has been demonstrated by Aldefluor assay (Yokota et al., 2009, Denning et al., 2011, Jaensson-Gyllenback et al., 2011, Cerovic et al., 2012). The assay reagent comprises an aminoacetaldehyde moiety bound to a boron-dipyromethene (BODIPY) fluorochrome, which passively diffuses into cells. As a member of the aldehyde dehydrogenase (ALDH) family of enzymes, RALDH oxidises the aminoacetaldehyde moiety and converts it to aminoacetate. The net negative charge of the aminoacetate moiety then prevents exit by diffusion, leading to an accumulation of intracellular BODIPY and increased cellular fluorescence which is detectable by flow cytometry (Storms et al., 1999). Furthermore, the assay is performed in the presence of the efflux inhibitor verapamil in order to prevent loss of fluorescence by active efflux of the oxidised reagent. The Aldefluor reaction mechanism is summarised in Figure 3.1.

The Aldefluor assay was originally developed for the identification and isolation of hematopoietic stem cells based on high aldehyde dehydrogenase (ALDH) activity and a

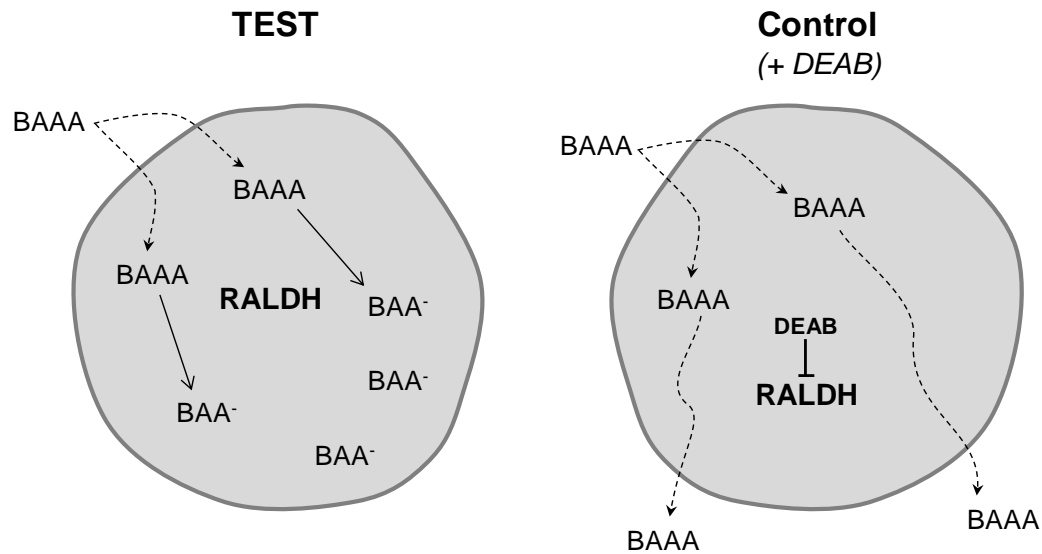


Figure 3.1: Mechanism of the Aldefluor assay. BODIPY aminoacetaldehyde (BAAA) diffuses into 'Test' cells and is oxidised by ALDH enzymes including the RALDH family to BODIPY aminoacetate (BAA⁻), which is prevented from exiting the cell by passive diffusion due to its net negative charge. Active efflux is also inhibited by the presence of verapamil in the assay buffer. This leads to an accumulation of fluorescence (520 – 540 nm) which is detectable by flow cytometry. In 'Control' cells, DEAB prevents the oxidation of BAAA to BAA⁻ by RALDH, preventing the accumulation of fluorescence. Dotted arrows indicate movement of reagents across the cell membrane, solid arrows indicate oxidation.

low side scatter profile (Storms et al., 1999). It has since been used to study cancer stem cells in a range of different contexts, where high ALDH activity is an indicator of poor prognosis (Alison et al., 2010). However, its use in identifying RALDH activity in human intestinal DCs has not yet been reported.

This chapter focuses on determining the suitability of the Aldefluor assay in determining RALDH activity within human intestinal DCs. These cells are obtained at relatively low frequencies following collagenase digestion of intestinal mucosa, therefore these experiments were performed on monocyte-derived DCs (MoDCs) generated *in vitro* with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). Aldefluor activity of human

MoDCs has been reported (Villablanca et al., 2011b), whilst expression of *ALDH1A2* has also been identified in these cells (Kamada et al., 2009). However, the relationship between RALDH and Aldefluor activity in MoDCs remains unaddressed.

The MoDC system also allows the influence of exogenous factors on RALDH activity to be studied. RA has been proposed as a key inducer of RALDH activity in intestinal DCs (Molenaar et al., 2011, Villablanca et al., 2011b). Furthermore, TLR stimulation exerts some influence over RALDH activity (Wang et al., 2011b, Villablanca et al., 2011b). Following validation of the Aldefluor assay in MoDCs, this system therefore allows the nature of this regulation to be explored in more detail.

3:3 Aims

1. Determine whether the Aldefluor assay specifically identifies RALDH activity in human DCs
2. Identify *ALDH1A* isoforms that drive Aldefluor activity in human DCs
3. Identify factors which influence RALDH activity in human DCs

3:4 Results

3:4.1 Human MoDCs have RALDH-specific Aldefluor activity

MoDCs were differentiated from CD14⁺ monocytes in the presence of GM-CSF and IL-4. Following the Aldefluor assay, a variable proportion of MoDCs displayed enhanced fluorescence consistent with intracellular accumulation of the acetate form of the Aldefluor reagent. In contrast, the accumulation of fluorescence (Aldefluor activity) was reduced in the presence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB, Figure 3.2A). This indicates activity of the ALDH family of enzymes within human MoDCs.

Aldefluor activity of MoDCs was inhibited in the presence of 10 μ M retinal. However, this inhibition was also present with concentration-matched vehicle control (DMSO; Figures 3.2B and C), indicating a suppressive effect by DMSO. Almost complete suppression of Aldefluor activity was observed with 100 μ M retinal which was not observed with concentration-matched DMSO (Figures 3.2B and C). This indicates that at sufficient molar excess (100 μ M versus 1.5 μ M for retinal and Aldefluor reagent, respectively) retinal competes with the Aldefluor reagent for access to the active site of ALDH enzymes. This strongly suggests that the observed Aldefluor activity in MoDCs is due to the RALDH (ALDH1A) sub-group of ALDH enzymes which specifically convert retinal into RA.

The specific RALDH isoforms responsible for the observed Aldefluor activity in human MoDCs were unknown. To address this, Aldefluor^{hi} and Aldefluor^{lo} MoDCs were sorted by flow cytometry immediately following the Aldefluor assay and *ALDH1A* expression in each subset was determined by real-time PCR. To determine the sorting efficiency, aliquots of both subsets were also analysed by flow cytometry post-sorting (Figure

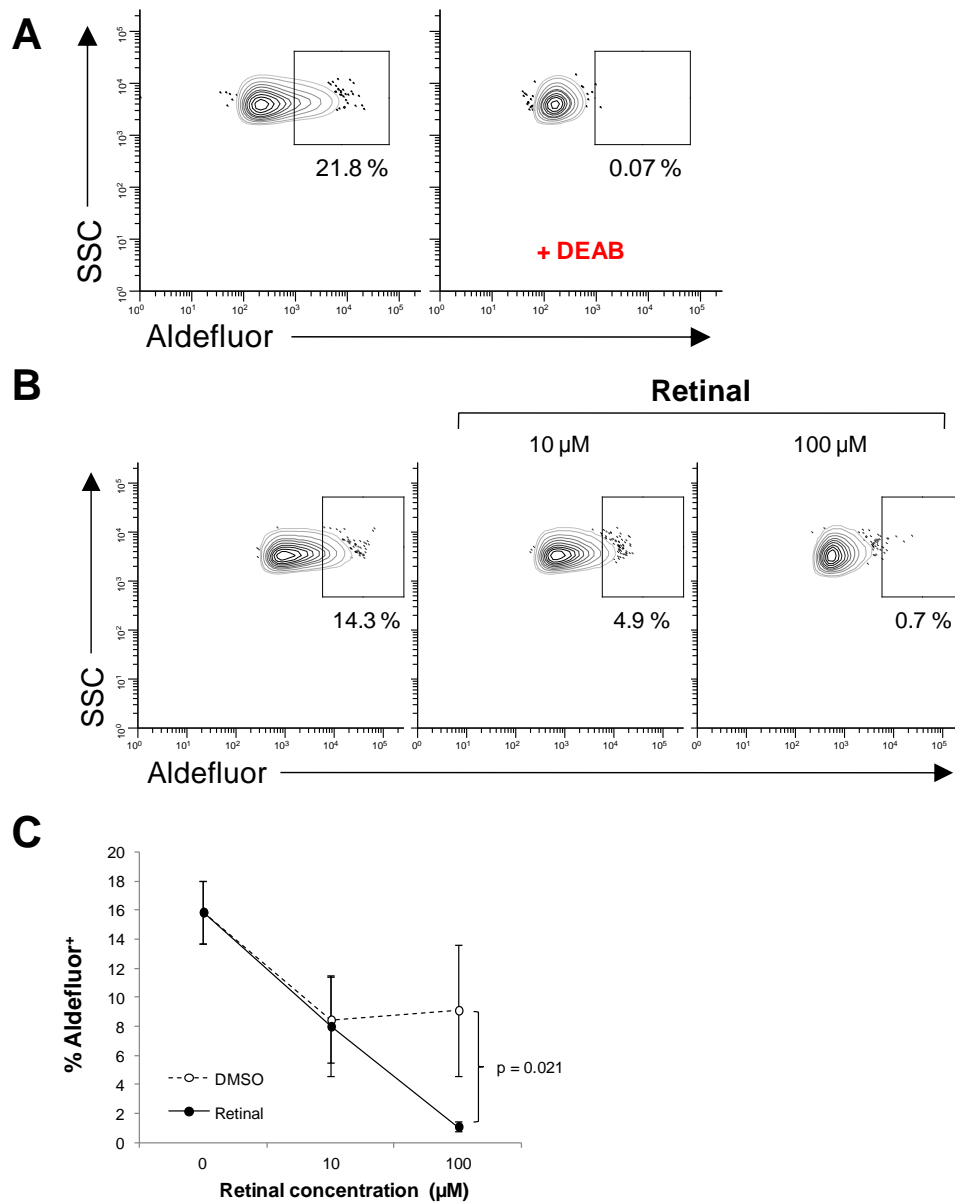


Figure 3.2: Human MoDCs possess Aldefluor activity that is specifically inhibited by retinal. **A:** CD14⁺ monocytes were cultured in the presence of GM-CSF and IL-4 (100 ng/ml) for 7 days to generate MoDCs which were then analysed by Aldefluor assay. Representative Aldefluor activity of MoDCs (n=16); the proportion of Aldefluor^{hi} cells is indicated and is defined relative to a DEAB-inhibited control sample (right). **B:** Representative example of Aldefluor assay performed on MoDCs in the presence or absence of retinal. **C:** Summary data of MoDC Aldefluor data from two independent experiments performed in duplicate with concentration-matched vehicle control (DMSO). Mean values displayed \pm SD; one way ANOVA with pair-wise comparison by Tukey test.

3.3A). The Aldefluor^{lo} subset was highly pure; however the Aldefluor^{hi} subset contained both Aldefluor^{hi} and Aldefluor^{lo} cells. This most likely reflects loss of the Aldefluor reagent over time during the sorting process rather than a low sorting efficiency for this subset.

ALDH1A1, *ALDH1A2* and *ALDH1A3* were expressed at approximately 2, 9 and 10 fold greater levels in Aldefluor^{hi} compared with Aldefluor^{lo} MoDCs, respectively (Figure 3.3B). *ALDH1A3* was expressed at levels considerably lower than those of *ALDH1A1* or *ALDH1A2*. This observation indicates that all three major RALDH isoforms could contribute to the observed Aldefluor activity of human MoDCs. At the level of transcription, RALDH2 appears to be the predominant enzyme in this context.

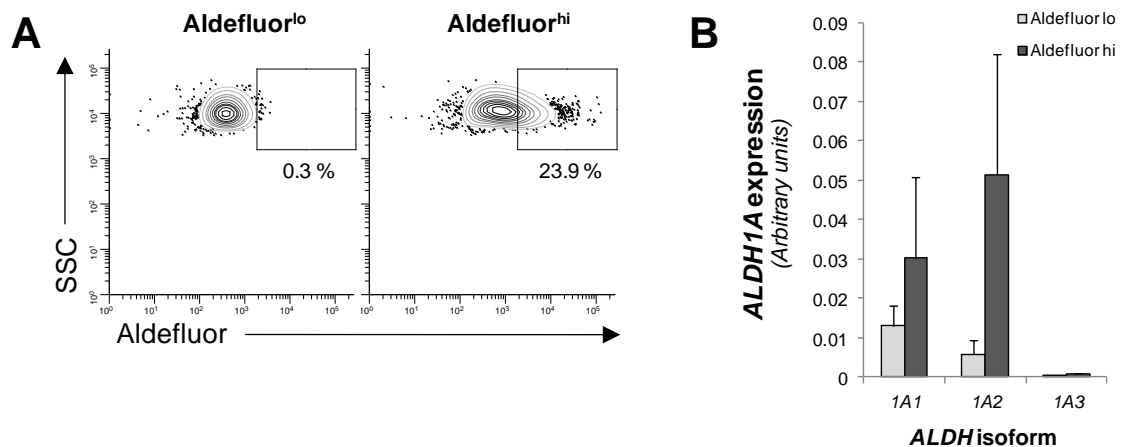


Figure 3.3: Human MoDCs with high levels of Aldefluor activity have enhanced *ALDH1A* expression. **A:** CD14⁺ monocytes were cultured in the presence of GM-CSF and IL-4 (100 ng/ml) for 7 days to generate MoDCs and then sorted by flow cytometry on the basis of Aldefluor activity. Representative example (n=3) of post-sort Aldefluor^{lo} and Aldefluor^{hi} MoDCs. **B:** Summary data of *ALDH1A* expression in flow-sorted Aldefluor^{lo} and Aldefluor^{hi} MoDCs as determined by real-time PCR (n=3); data normalized to *GAPDH*; mean values displayed + SD.

3:4.2 RA is both required for and enhances RALDH activity of human MoDCs

It has been demonstrated that dietary vitamin A (retinol) contributes significantly to the RALDH activity of murine intestinal CD103⁺ DCs (Molenaar et al., 2011, Jaensson-Gyllenback et al., 2011). Furthermore, exogenous RA boosts Aldefluor activity in human MoDCs (Villablanca et al., 2011b). This likely represents a positive feedback loop, although the mechanism behind this remains undetermined. It is also currently unknown how the presence or absence of RA during the differentiation process influences RALDH activity of human MoDCs. Monocytes were therefore cultured in GM-CSF and IL-4 with the addition of exogenous RA or the RAR α antagonist Ro41-5253 (Apfel et al., 1992) in order to block endogenous RA present in FCS or generated by DC themselves. The Aldefluor/RALDH activity of control MoDCs was consistently enhanced by the presence of 10 nM RA across 3 independent experiments, although this did not reach statistical significance (Figures 3.4A and B). Furthermore, Ro41-5253 suppressed the Aldefluor/RALDH activity of MoDCs (Figures 3.4A and B). Overall there was a statistically significant increase in the RALDH/Aldefluor activity of RA versus Ro41-5253-treated MoDCs ($p = 0.002$; Figure 3.4B). This indicates that RALDH activity responsible for Aldefluor activity in human MoDCs requires the presence of RAR α signalling during differentiation, and that the activity of these enzymes may be further upregulated by exogenous RA.

Of the three major RALDH isoforms analysed by real-time PCR, only expression of *ALDH1A2* was enhanced by the addition of RA as compared with control MoDCs (Figure 3.4C). This increase did not reach statistical significance across three independent experiments, and may indicate a role for post-transcriptional regulation of *ALDH1A2* in determining overall RALDH2 activity. In contrast, *ALDH1A1* expression of

MoDCs was significantly suppressed by the presence of exogenous RA ($p = 0.002$; Figure 3.4C). Whilst *ALDH1A3* was generally expressed at a significantly lower level than the other isoforms, it too was reduced by RA ($p = 0.077$; Figure 3.4C). Overall, this indicates that the enhanced Aldefluor activity of MoDCs grown in the presence of exogenous 10 nM RA is the partially the result of enhanced *ALDH1A2* expression, with a potential additional role for post-translational regulation. Based on reduced

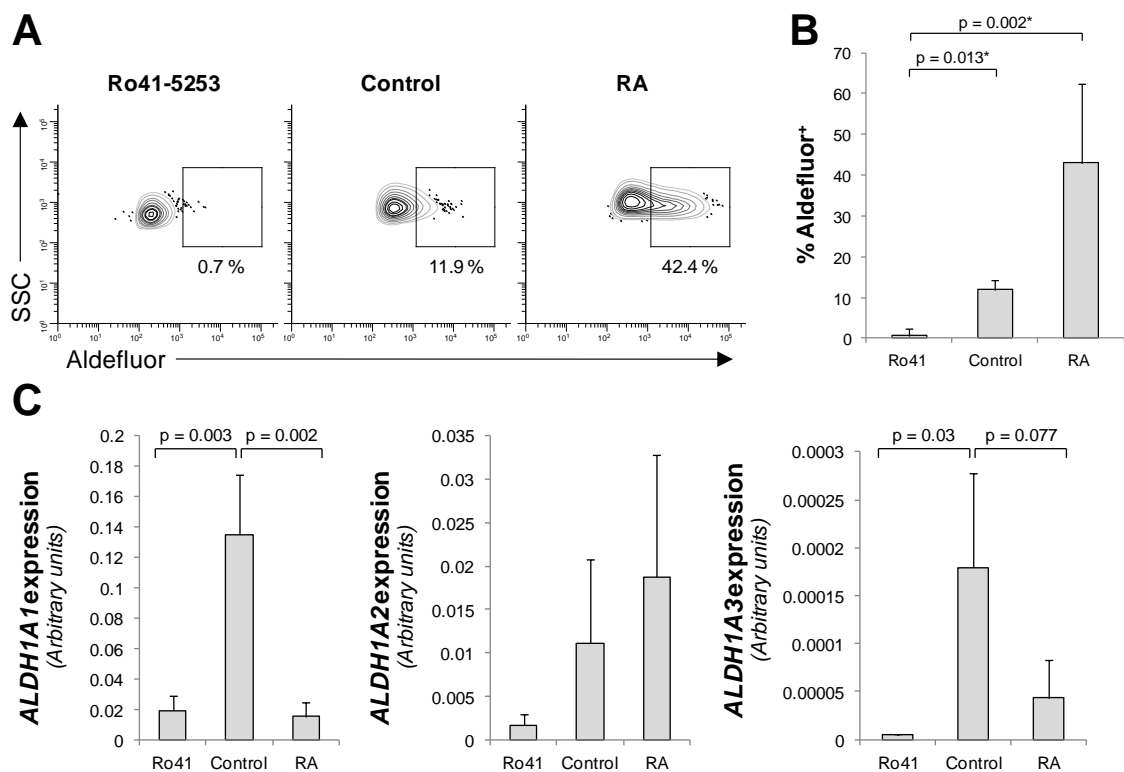


Figure 3.4: Retinoic acid is both required for and enhances RALDH2-mediated Aldefluor activity of human MoDCs. **A:** CD14⁺ monocytes were cultured with GM-CSF and IL-4 (100 ng/ml) for 7 days to generate MoDCs in the presence of Ro41-5253 (1 μ M), RA (10 nM) or neither (control). Representative example ($n=3$) of Aldefluor activity of MoDC groups; numbers the proportion of Aldefluor^{hi} cells gating relative to a DEAB control (not shown). **B:** Summary Aldefluor data ($n=3$). **C:** *ALDH1A* expression determined in treated MoDC groups by real-time PCR ($n=3$), expression normalized to *GAPDH*. Mean values displayed + SD except in B (median); one way ANOVA with pair-wise comparison by Tukey test; * indicates p -value following \log_{10} transformation of non-normally distributed data.

transcription, neither *RALDH1* nor *RALDH3* are likely to contribute to this enhanced activity.

ALDH1A1 and *ALDH1A3* expression were suppressed by the presence of Ro41-5253 during MoDC differentiation ($p = 0.003$ and $p = 0.03$, respectively; Figure 3.4C). *ALDH1A2* expression was also suppressed, although this did not reach statistical significance (Figure 3.4C). This indicates that RAR α signalling is required for the expression of all major RALDH isoforms in human MoDCs, and that each may contribute to the overall Aldefluor activity of these cells as suggested earlier (Figure 3.3B). However, the effect of RA at a concentration of 10 nM on the expression of *ALDH1A2* is distinct from its effect on *ALDH1A1* and *ALDH1A3*, suggesting that exposure to RA may regulate the balance of *ALDH1A* isoform expression in human DCs.

3:4.3 RALDH activity and *ALDH1A* expression of human MoDCs is influenced by maturation status

It has been demonstrated in mice that RALDH activity may be induced in splenic DCs by stimulation of TLR1/2 with the bacterial lipoprotein analogue Pam3CSK4 (Lee et al., 2012). Furthermore, murine intestinal CD103⁺ DCs display reduced RALDH activity in MyD88^{-/-} mice in which signalling through multiple TLRs (as well as IL-1) is suppressed (Wang et al., 2011b). Pam3CSK4 also induces *ALDH1A2* expression in human MoDCs (Wang et al., 2011b). However, the extent to which modulation of RALDH activity following TLR stimulation is related to DC maturation status has not been previously explored. To address this, Aldefluor/RALDH activity of human MoDCs was assessed following overnight culture in the presence or absence of LPS (1 μ g/ml). Control MoDCs existed in a range of states from immature (HLA-DR^{lo} CD86⁻) to mature (HLA-

DR^{hi}CD86⁺). In contrast, following culture with LPS MoDCs displayed a uniformly mature phenotype (Figure 3.5A). LPS-treated MoDCs consistently displayed increased levels of Aldefluor/RALDH activity across three independent experiments (Figures 3.5B and C). Increased Aldefluor/RALDH activity was associated with a trend towards increased expression of both *ALDH1A2* and *ALDH1A3*, with decreased expression of *ALDH1A1* (Figure 3.5D). Unstimulated MoDCs with a mature phenotype (HLA-DR^{hi}CD86⁺) display significantly increased Aldefluor/RALDH activity compared with their immature (HLA-DR^{lo} CD86⁻) counterparts ($p = 0.005$; Figures 3.5E and F). Overall, this indicates that RALDH activity of human MoDCs is largely restricted to subset of cells with a mature phenotype. The ability of LPS to increase Aldefluor/RALDH activity of MoDCs appears to relate directly to increasing the proportion of cells with this phenotype.

3:4.4 GM-CSF induces RALDH activity in human MoDCs

It has been demonstrated that the combination of GM-CSF and IL-4 is sufficient to induce RALDH activity in splenic DCs in mice (Yokota et al., 2009). GM-CSF alone induces significant levels of RALDH activity in non-intestinal DCs in mice following administration of adenovirus 5, leading to peripheral induction of $\alpha_4\beta_7^+$ CD8⁺ T cells (Ganguly et al., 2011b). The individual contribution of GM-CSF and IL-4 to the Aldefluor/RALDH activity of human MoDCs is unknown. To address this, monocytes were differentiated either in the presence of both GM-CSF and IL-4 as standard (control), or else with GM-CSF or IL-4 alone. Monocytes cultured with IL-4 alone displayed very low levels of Aldefluor/RALDH activity (Figures 3.6A and B). In contrast, monocytes cultured with GM-CSF alone had significantly elevated Aldefluor/RALDH

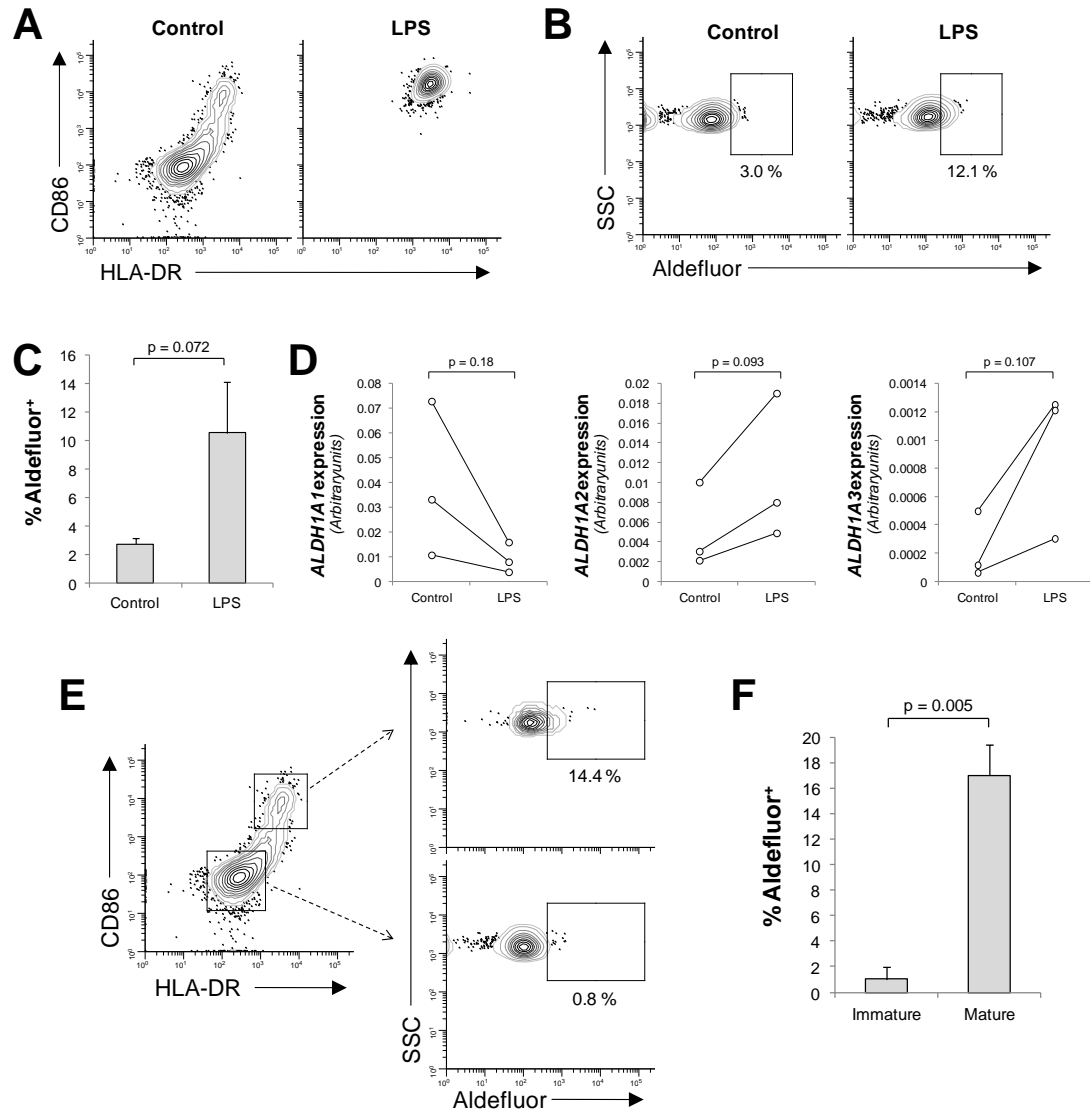


Figure 3.5: RALDH activity of human MoDCs is affected by maturation status. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days to generate MoDCs, which were then cultured overnight in the presence or absence of LPS (1 μ g/ml) and CD86/HLA-DR expression determined by flow cytometry as an indication of maturation state, representative example (n=3). **B:** Representative example (n=3) of Aldefluor/RALDH activity of control and LPS-treated MoDCs. **C:** Summary Aldefluor/RALDH activity of control and LPS-treated MoDCs (n=3). **D:** Expression of *ALDH1A* isoforms was determined in control and LPS-treated MoDCs by real time PCR (n=3); normalized to *RPL30*. **E:** Representative example of Aldefluor/RALDH activity of mature and immature control MoDCs. **F:** Summary Aldefluor/RALDH activity of mature and immature control MoDCs (n=3). Mean values displayed + SD; paired t-tests used throughout.

activity as compared with control MoDCs and monocytes grown with IL-4 ($p = 0.018$ and $p = 0.008$, respectively; Figures 3.6A and B). Overall, this indicates that GM-CSF is the principal driving factor behind Aldefluor/RALDH activity in human MoDCs, and that IL-4 acts as a negative RALDH factor in this context.

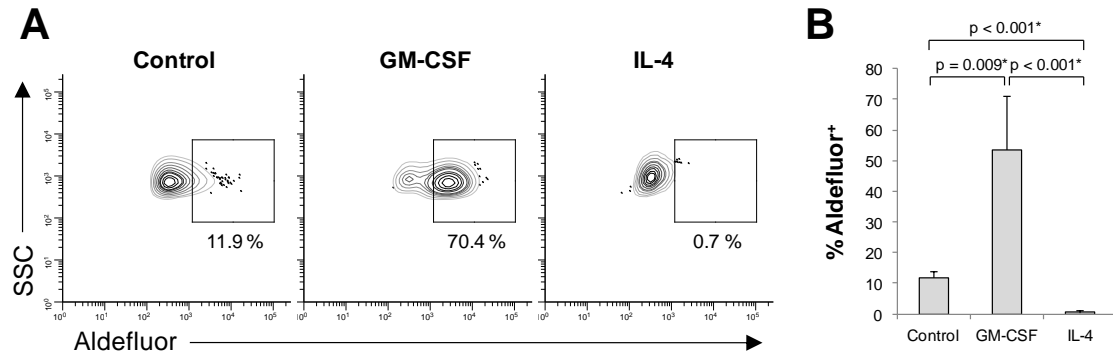


Figure 3.6: GM-CSF alone induces significantly greater levels of Aldefluor activity in MoDCs than GM-CSF in combination with IL-4. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (Control) or GM-CSF or IL-4 alone (all at 100 ng/ml) for 7 days and analysed by Aldefluor assay; representative data (n=3). **B:** Summary Aldefluor data (n=3); median values displayed + SD; one way ANOVA with pair-wise comparison by Tukey test; * indicates p-value following log₁₀ transformation of non-normally distributed data.

3:5 Discussion

The primary aim of the work presented in this chapter was to establish the validity of the Aldefluor assay in analysis of RALDH activity of human MoDCs, with a view to extending this to intestinal DCs. MoDCs were used in these experiments as they could be generated under controlled conditions and in quantity sufficient for analysis. They displayed an accumulation of the Aldefluor reagent which could be blocked with DEAB and competitively inhibited with retinal. Furthermore, cells with increased Aldefluor activity had increased expression of all *ALDH1A* isoforms studied. Overall, this indicates a significant overlap between Aldefluor and RALDH activity in human MoDCs.

The presence of exogenous 10 nM RA throughout differentiation increased the level of Aldefluor/RALDH activity of MoDCs upon analysis at day 7. Addition of RA on day 3 of differentiation has also been demonstrated to increase Aldefluor activity of human MoDCs (Villablanca et al., 2011b). This indicates that the positive feedback loop between RA and RALDH enzyme activity is not restricted to an early stage of differentiation, but may be amplified throughout this process.

The mechanism of enhanced Aldefluor/RALDH activity through exogenous RA remains unclear. Of the three isoforms studied, only expression of *ALDH1A2* was increased by this treatment whereas *ALDH1A1* and *ALDH1A3* were suppressed. This implicates RALDH2 activity in this context, though the relatively small increase in *ALDH1A2* expression also suggests post-transcriptional regulation. This has been proposed elsewhere to account for a diminishing gradient of Aldefluor/RALDH activity in murine intestinal DCs from the proximal to distal intestine, despite relatively stable *Aldh1a2* expression by DCs across the murine intestine (Villablanca et al., 2011b). Furthermore, a precedent exists for RA and RAR α influencing the post-transcriptional modification of

proteins. In the presence of RA, RAR α promotes translation of the glutamate receptor GluR1 in rat primary hippocampal neurons (Maghsoodi et al., 2008). RA may therefore support translation of *ALDH1A2* mRNA in human MoDCs.

The mechanisms regulating RA-mediated transcriptional control of *ALDH1A2* remain unclear. The murine *Aldh1a2* promoter does not contain RARE sites, suggesting that transcriptional regulation is indirect and not by direct promoter binding of RARs (Wang et al., 2001). Administration of RA has been shown to lead to phosphorylation of the MAPK ERK1/2, and this activation is required for the induction of RALDH in murine splenic DCs by RA (Villablanca et al., 2011b). In a different context, *Rara*^{-/-} mice (deficient in RAR α) display defects in T cell activation and proliferation which associates with reduced phosphorylation of ERK1/2 (Hall et al., 2011b). Signalling events downstream of ERK1/2 may therefore dictate enhanced *ALDH1A2* expression following administration of exogenous RA.

Expression of all *ALDH1A* isoforms was suppressed in MoDCs by inhibition of RAR α signalling with Ro41-5253 during MoDC differentiation. This indicates that RA is required for expression of RA-generating enzymes and that the estimated 0.2 – 0.5 nM RA present in complete medium containing 10% FCS (Kang et al., 2011) is sufficient to support this expression. *ALDH1A* expression may also be supported by low levels of RA production by MoDCs in a positive feedback loop. This interpretation is supported by the presence of a non-consensus RARE in the human *ALDH1A1* promoter that recruits RAR α /RXR β which then acts in conjunction with the CCAAT enhancer binding protein (C/EBP)- β to promote transcription of *ALDH1A1* (Elizondo et al., 2000). Interestingly, exogenous RA suppresses binding of C/EBP β to CCAAT (Elizondo et al., 2009), providing an explanation for suppression of *ALDH1A1* in human MoDCs grown in the presence of 10 nM RA. Reduced C/EBP β binding may be the result of reduced

transcription and/or the presence of additional proteins such as GADD153 which bind to C/EBP β and reduce its ability to interact with CCAAT sites (Elizondo et al., 2009). *ALDH1A3* expression may be regulated in a similar manner, as it too was suppressed by both Ro41-5253 and RA. The murine *Aldh1a2* promoter contains CCAAT sites (Wang et al., 2001), but may be regulated by RA within a distinct range of concentrations. Regulation of *ALDH1A* expression by RA also varies between distinct cell types. It has been demonstrated that high dose RA (1 μ M) significantly enhances *ALDH1A3* expression in human keratinocytes, whilst *ALDH1A1* and *ALDH1A2* are suppressed (Koenig et al., 2010).

It is interesting to note that within a single population of MoDCs, Aldefluor/RALDH activity was largely limited to cells with a mature phenotype. Whether the upregulation of RALDH activity is required for or is simply co-incident with DC maturation is unclear. It has been demonstrated that a combination of RA and TNF α is required to mature a Langerhans cell (LC)-type DC, a modified MoDC in which TGF- β is present during culture (Geissmann et al., 2003). This study also observed that 1 μ M retinol induced apoptosis in immature but not mature LC-type DCs during two day culture. This may indicate that the RALDH activity of mature DCs contributes to metabolism of retinal which may otherwise accumulate during this culture period, leading to the apoptosis of immature DCs.

LPS stimulation did not significantly increase the Aldefluor/RALDH activity of human MoDCs beyond increasing the proportion of cells with a mature phenotype. Stimulation of human MoDCs with the TLR2 ligand Pam3CSK4 increases *ALDH1A2*, as reported elsewhere (Villablanca et al., 2011b), and is consistent with an association with maturation-inducing stimulation and upregulation of RALDH activity. Moreover, stimulation of murine splenic DCs with LPS, Pam3CSK4 or flagellin enhances

expression of *Aldh1a2* (Wang et al., 2011b). In contrast with RA-mediated RALDH upregulation, this requires the MAPK JNK and not ERK1/2. The extent to which these stimulations upregulate RALDH as part of DC maturation is however unclear.

The significantly enhanced ability of GM-CSF to induce Aldefluor/RALDH activity in human monocytes compared with GM-CSF in combination with IL-4 was unexpected, as GM-CSF and IL-4 synergistically induce RALDH activity in murine splenic DCs (Yokota et al., 2009). Monocytes cultured in GM-CSF alone differentiate into a classically activated or 'M1' M Φ phenotype (Krausgruber et al., 2011), as opposed to a MoDC phenotype. These cells display a pro-inflammatory phenotype characterized by high production of TNF α and low amounts of IL-10 in response to microbial stimulation (reviewed by Hedl and Abraham, 2013). The relationship between RALDH activity and a pro-inflammatory M Φ phenotype is explored in detail in Chapter 5. The ability of GM-CSF to induce RALDH activity human CD14⁺ monocytes is consistent with GM-CSF-mediated induction of RALDH activity in peripheral DCs in mice following intra-muscular administration of adenovirus type 5 (Ganguly et al., 2011b). This also suggests that immunological contexts in which GM-CSF production is induced in humans may lead to peripheral induction of DC RALDH activity and the potential induction of gut-homing T cell responses from these sites. IL-4 alone was a poor inducer of Aldefluor/RALDH activity in human monocytes, and reduced the activity induced by GM-CSF alone. In contrast, IL-4 in conjunction with Flt3L induces *Aldh1a2* expression in murine bone marrow (BM)-derived DCs (Yokota et al., 2009). Furthermore, IL-4 also induces *Aldh1a2* expression murine BM-derived M Φ in conjunction with M-CSF (Broadhurst et al., 2012). Thus, the effect of IL-4 on RALDH activity in DCs may be dependent upon the context provided by additional cytokines or alternatively this property may be specific to IL-4 in the mouse.

3:6 Conclusion

In conclusion, Aldefluor assay is a valid approach for determining RALDH activity in human MoDCs. In the next chapter, this methodology is applied to DCs extracted from human intestinal mucosa in order to identify which population have RALDH activity and to investigate whether this activity is altered in IBD.

Chapter 4: Analysis of antigen presenting cell retinaldehyde dehydrogenase activity and retinoid levels in the healthy human intestine and in inflammatory bowel disease

4:1 Chapter summary

This chapter focuses on determining the distribution of RALDH activity in human intestinal antigen presenting cell (APC) subsets in healthy control and IBD patients using the Aldefluor assay as validated in Chapter 3. RALDH activity is identified in intestinal CD103⁺ and CD103⁻ DCs, and also CD14⁺ MΦ. This activity derives from differential expression of *ALDH1A* isoforms, with CD14⁺ MΦ distinguished by expression of *ALDH1A1*. RALDH activity of CD103⁺ DCs is not reduced in IBD patients, arguing against a hypothesis that loss of this activity contributes to disease pathogenesis. In contrast, this activity is slightly increased in IBD and may therefore mediate currently unknown aspects of disease. RALDH activity of CD14⁺ MΦ is significantly enhanced in IBD patients. RALDH activity is also observed in non-haematopoietic cells, including intestinal epithelial cells, which is reduced in IBD patients. Preliminary data suggests that net levels of RA are maintained across healthy control and CD intestinal mucosa, despite a reduction in the level of mucosal retinol. In conclusion, this chapter provides the first detailed analysis of RALDH activity in APC subsets in the human intestinal mucosa in health and IBD. It identifies key differences between mouse and man which may ultimately provide critical insight into the pathogenesis of IBD.

4:2 Introduction

A paradigm has emerged over recent years in which CD103⁺ DCs play a key role in supporting intestinal homeostasis in mice. It is now well established that CD103⁺ DCs in the murine small intestinal lamina propria (SI-LP) have enhanced levels of RALDH activity compared with CD103⁻ DCs (Schulz et al., 2009, Denning et al., 2011, Jaensson-Gyllenback et al., 2011). This is also true in MLNs (Coombes et al., 2007, Yokota et al., 2009, Molenaar et al., 2011, Guilliams et al., 2010) where CD103⁺ DCs are believed to have migrated from the SI-LP via the draining lymphatics (Johansson-Lindbom et al., 2005, Jaensson et al., 2008). Enhanced RALDH activity is reflected in high level expression of *Aldh1a2*, encoding RALDH2, in CD103⁺ DCs (Coombes et al., 2007, Schulz et al., 2009, Yokota et al., 2009, Denning et al., 2011). RALDH activity has also been observed in murine colonic LP CD103⁺ DCs, albeit at lower levels than in the SI-LP (Denning et al., 2011, Jaensson-Gyllenback et al., 2011, Villablanca et al., 2011b).

Provision of RA by murine CD103⁺ DCs during lymphocyte activation imprints a 'gut-homing' $\alpha_4\beta_7^+$ CCR9⁺ phenotype on T cells (Iwata et al., 2004, Johansson-Lindbom et al., 2005, Jaensson et al., 2008) and similar pathways operate in B cells (Mora et al., 2006). This allows efficient entry of activated T and B cells into the intestinal mucosa from blood circulation. RA derived from CD103⁺ DCs enhances TGF- β -mediated induction of Foxp3⁺ T_{REG} (Sun et al., 2007, Coombes et al., 2007) as well as suppressing induction of T_H17 cells (Mucida et al., 2007). Furthermore, CD103⁺ DCs use RA to drive the differentiation of IgA⁺ plasma cells from naive B cells (Mora et al., 2006, Uematsu et al., 2008).

Human MLN CD103⁺ DCs have been shown to be unique in their ability to induce high levels of CCR9 expression on naïve CD8⁺ T cells (Jaensson et al., 2008), although RALDH activity in these cells has not been addressed. Whilst this indicates a degree of overlap between mouse and man, the distribution and role of RALDH activity in human intestinal CD103⁺ and CD103⁻ DCs remain poorly defined.

Mouse models of IBD indicate that RALDH activity in CD103⁺ DCs is significantly modified under inflammatory conditions. TNFΔARE mice, in which the stability of TNFα mRNA is enhanced, develop chronic inflammation of the terminal ileum (TI) with similar histology to Crohn's disease (Kontoyiannis et al., 1999). Expression of *Aldh1a2* is significantly reduced in CD103⁺ DCs in the TI LP of TNFΔARE mice by 4 weeks of age, and then in the MLN by 20 weeks. This may contribute to inflammation by altering the balance of T cell profiles induced by CD103⁺ DCs in favour of T_H17 cells over Foxp3⁺ T_{REG} (Collins et al., 2011). Furthermore, *Aldh1a2* expression is significantly reduced in MLN CD103⁺ DCs in the T cell transfer model of colitis (Laffont et al., 2010). CD103⁺ DCs from these mice display a reduced ability to induce Foxp3⁺ T_{REG}, with enhanced induction of both T_H1 and T_H17 cells. Whether RALDH activity is lost in human CD103⁺ DCs in IBD and the extent to which this contributes to the pathogenesis of disease is currently unknown.

Beyond DCs, RALDH activity has also been identified in CD11b⁺ CD11c⁻ F4/80⁺ resident MΦ in both SI and colonic LP of mice, along with expression of *Aldh1a1* (Denning et al., 2011). In the presence of exogenous TGF-β, these cells induce differentiation of naïve CD4⁺ T cells into Foxp3⁺ T_{REG} *in vitro* which may be inhibited by blocking either IL-10 or RA (Denning et al., 2007). They have also been implicated in the expansion of Foxp3⁺ T_{REG} within the LP required for the induction of oral tolerance (Hadis et al., 2011). In humans, RALDH activity has not been systematically examined

but *ALDH1A2* expression has been identified in intestinal CD14⁺ MΦ (Kamada et al., 2009). These cells are a pro-inflammatory population (Kamada et al., 2008) and production of RA by these cells may influence the induction of T_H17 cells (Kamada et al., 2009). As with DCs, the nature of RALDH activity in human CD14⁺ DCs is poorly defined.

In this chapter, multi-colour flow cytometry was used to identify CD103⁺ and CD103⁻ DCs as well as CD14⁺ MΦ in lamina propria cells (LPCs) extracted from human intestinal mucosa. RALDH activity was assessed in these populations using the Aldefluor assay as validated in Chapter 3. Expression of *ALHD1A* enzyme isoforms were assessed in purified cell populations obtained by cell sorting through flow cytometry. Furthermore, RALDH activity of these APC subsets was compared across healthy control and IBD patients in order to establish whether these are regulated by intestinal inflammation. Finally, mucosal levels of RA were analysed in order to determine whether observed changes in RALDH activity are sufficient to influence the local retinoid environment. Significant changes in RALDH activity in these subsets may provide key insights into the pathogenesis of IBD, and ultimately provide fresh therapeutic options.

The hypothesis for this chapter was therefore that RALDH activity is enhanced in CD103⁺ DCs within healthy human intestinal mucosa as compared with either CD103⁻ DCs or CD14⁺ MΦ. This would reflect a parallel distribution of RALDH activity as observed in the mouse. Furthermore, this activity would be reduced in patients with IBD, consistent with both TNFΔARE (Collins et al., 2011) and T cell transfer-mediated (Laffont et al., 2010) murine intestinal inflammation. Loss of RALDH activity may lead to reduced induction of FOXP3⁺ T_{REG} and/or a modified ability to induce a gut-homing α₄β₇⁺ phenotype on naive CD4⁺ T cells during activation, which may contribute to the

pathogenesis of IBD. Loss of RALDH activity may also lead to a reduced overall concentration of RA within the intestinal mucosa as implicated in TNF Δ ARE mice (Collins et al., 2011).

4:3 Aims

1. Determine which APC populations in human intestinal mucosa have RALDH activity
2. Define changes in RALDH activity that occur in IBD
3. Compare retinoid concentrations in healthy and IBD intestinal mucosa

4:4 Results

4:4.1 RALDH activity is detected in multiple human intestinal APC subsets

The ultimate aim of this project was to determine whether alterations in the RALDH activity present in human intestinal APCs plays a role in the pathogenesis of IBD and whether modification of this pathway has a potential therapeutic role. There is currently a significant gap between what is understood in the murine and human intestinal immune systems. For any observed differences in RALDH activity in IBD to have meaning, extensive characterisation of the distribution and function of RALDH activity in human intestinal APCs was therefore required.

The first step taken was the identification of human intestinal APC subsets via flow cytometry. Lamina propria cells (LPCs) were extracted from mucosa derived from patients undergoing colonic and/or ileal surgical resection due to cancer or functional bowel problems. In the case of cancer patients, there was a significant margin between affected areas and tissue used for this research. For the purposes of this study, this tissue was classified as healthy. LPCs extracted from collagenase digestion of this mucosa were analysed via flow cytometry, with a staining strategy biased towards myeloid DCs (Figure 4.1). Leukocytes were first selected as CD45⁺ cells, and then DCs were identified as Lineage⁻ (Lin: CD3, CD14, CD16, CD19, CD20, CD56) HLA-DR⁺ cells. Beyond this, myeloid DCs were identified as CD11c⁺ and could be further divided into CD103⁺ and CD103⁻ subsets.

There is ongoing controversy in the murine field as to the DC/macrophage (MΦ) nature of various intestinal APC subsets, but a clear distinction is the ability of DCs to upregulate the chemokine receptor CCR7 during maturation and thereby to migrate into

naive T cell areas in the draining MLNs (Jang et al., 2006, Schulz et al., 2009, Rivollier et al., 2012). Myeloid DCs analysed immediately following collagenase digestion of intestinal mucosa were CCR7⁻ (Figure 4.2A). However, when LPCs were cultured overnight at 37 °C to allow time for DC maturation in 3 independent experiments, the vast majority of CD103⁺ DCs expressed CCR7 (91.9 ± 2.5 %; Figure 4.2B). CD103⁻ DCs were also predominantly CCR7⁺ (65.6 ± 16.3 %), although this represented a slightly lower proportion of cells when compared with CD103⁺ DCs (Figures 4.2C and D). Overall these data strongly support the identification of CD103⁺ DCs as *bona fide* DCs, with a degree of heterogeneity amongst CD103⁻ DCs and the potential inclusion of more MΦ-like cells.

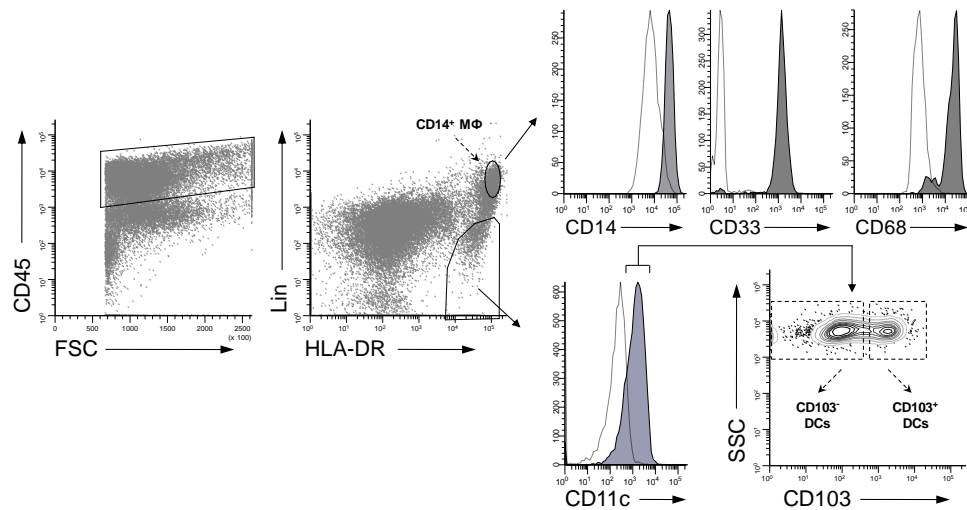


Figure 4.1: Identification of human intestinal APCs via flow cytometry. LPCs were extracted from collagenase-digested colonic and TI resections and APC subsets identified by flow cytometry. Gating was focussed on leukocytes (CD45⁺ cells); DCs were Lin⁻ HLA-DR⁺ and CD11c⁺ (myeloid cells), CD103⁺ and CD103⁻ DC subsets were defined by isotype control staining (not shown). Lin⁺ HLA-DR⁺ cells were also analysed, and defined as CD14⁺ MΦ based on expression of CD14, CD33 and CD68. Filled histogram represents antibody staining, dotted histogram represents the relevant isotype control; FSC is forward scatter, SSC is side-scatter.

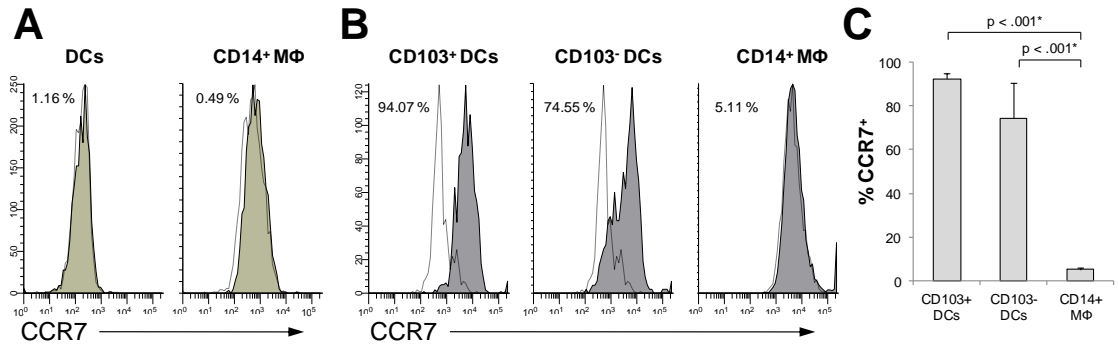


Figure 4.2: CD103⁺ and CD103⁻ DCs upregulate CCR7 during overnight culture, whilst CD14⁺ MΦ remain CCR7⁻. **A:** LPCs were extracted from collagenase-digested colonic biopsies, CCR7 expression was then immediately determined in DCs and CD14⁺ MΦ (as defined in Figure 4.1) via flow cytometry. **B:** LPCs were cultured overnight at 37 °C in complete medium; CCR7 expression was identified in APC subsets (as defined in Figure 4.1) via flow cytometry; filled histogram represents antibody staining, dotted histogram represents the relevant isotype control. The proportion of cells that are CCR7⁺ is indicated in each case. **C:** Summary data of CCR7 expression on APC subsets following overnight culture of LPCs obtained from healthy control biopsies (n=3). Bars indicate median values + SD; one way ANOVA with Tukey test; * indicates p-value obtained following log₁₀ transformation of transformation of non-normally distributed data.

As stated previously, the flow cytometry staining protocol was designed for the identification of human intestinal myeloid DC subsets. A discrete Lin^{bright}HLA-DR⁺ population was however also routinely identified (Figure 4.1). Previous work in the lab has attributed CD14 expression to these cells, contributing to the Lin^{bright} staining (Bell et al., 2001). Strong fluorescence was observed in these cells when labelled with the mouse Igγ2a isotype control antibody (control for anti-CD14 staining; Figure 4.1), indicating either a significant degree of autofluorescence in the Pacific Blue range of the fluorescence spectrum or else high levels of non-specific antibody binding. CD14 expression was however clearly observed in these cells beyond the isotype control staining. Furthermore, these cells also expressed CD33 and CD68 (Figure 4.1). Both are widely distributed on myeloid cells, but the presence of CD14 identifies these cells as CD14⁺ MΦ.

As with myeloid DCs, CD14⁺ MΦ analysed immediately following collagenase digestion of intestinal mucosa were CCR7⁻ (Figure 4.2A). However, following overnight culture of LPCs, the majority of cells remained CCR7⁻ across 3 independent experiments (Figures 4.2B and C). These data are consistent with CD14⁺ MΦ as a tissue-resident non-migratory MΦ population. Interestingly, a small proportion (5.5 ± 0.8 %) of CD14⁺ MΦ were CCR7⁺ in all experiments following culture, indicating heterogeneity within the CD14⁺ population and the possible existence of CD14⁺ MΦ with DC-like properties.

In order to characterise RALDH activity amongst these intestinal APC subsets, the Aldefluor assay was performed on LPCs and the activity was subsequently attributed to each subset via flow cytometry based on the gating strategy as defined in Figure 4.1. As seen in Figure 4.3A, CD103⁺ and CD103⁻ DCs as well as CD14⁺ MΦ possessed cells with enhanced Aldefluor/RALDH activity in comparison to the DEAB-inhibited controls.

The distribution of activity in these subsets was similar across 6 healthy control samples (Figure 4.3B); with mean values of 18.4, 18.6 and 22.3 % Aldefluor⁺ cells in CD103⁺ and CD103⁻ DCs and CD14⁺ MΦ, respectively. RALDH activity is enhanced in SI-LP CD103⁺ DCs in mice (Schulz et al., 2009, Denning et al., 2011, Jaensson-Gyllenback et al., 2011). Paired analysis of CD103⁺ and CD103⁻ DCs in this study however showed no enrichment of Aldefluor/ RALDH activity in human distal intestinal CD103⁺ DCs (Figure 4.3C).

Analysis of RALDH activity via the Aldefluor assay was then extended to intestinal lymphocytes in order to determine whether this was a general feature of human intestinal leukocytes. CD19⁺ B cells possessed low levels of Aldefluor/RALDH activity based on a small increase in Aldefluor signal compared with the DEAB control (Figure

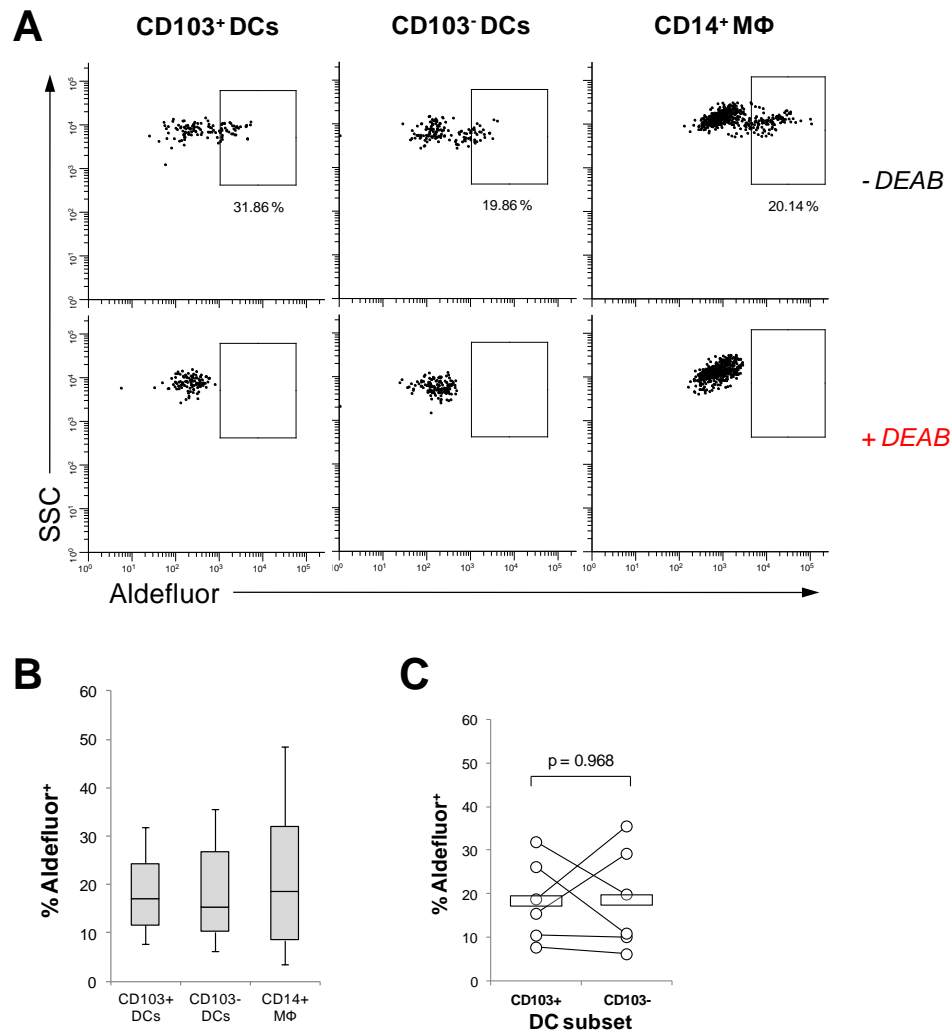


Figure 4.3: RALDH activity is identified in multiple intestinal APC subsets in healthy control patients. **A:** Aldefluor assay was performed in conjunction with flow cytometry on LPCs to identify CD103⁺ and CD103⁻ DCs and CD14⁺ MΦ from healthy control colon and TI resections with enhanced RALDH activity. Aldefluor⁺ gating was defined in comparison to a DEAB-inhibited control reaction (bottom row); a fixed separation was introduced between DEAB-inhibited cells and the gate to ensure only cells with enhanced RALDH activity were analysed. **B:** Box and whisker plot depicting the distribution of Aldefluor⁺ cells in each APC subset from healthy control samples (n=6). **C:** Paired analysis of the proportion of Aldefluor⁺ CD103⁺ and CD103⁻ DCs from healthy control intestinal mucosa. Lines indicate individual experiments, bars represent mean values; paired t-test. SSC is side-scatter.

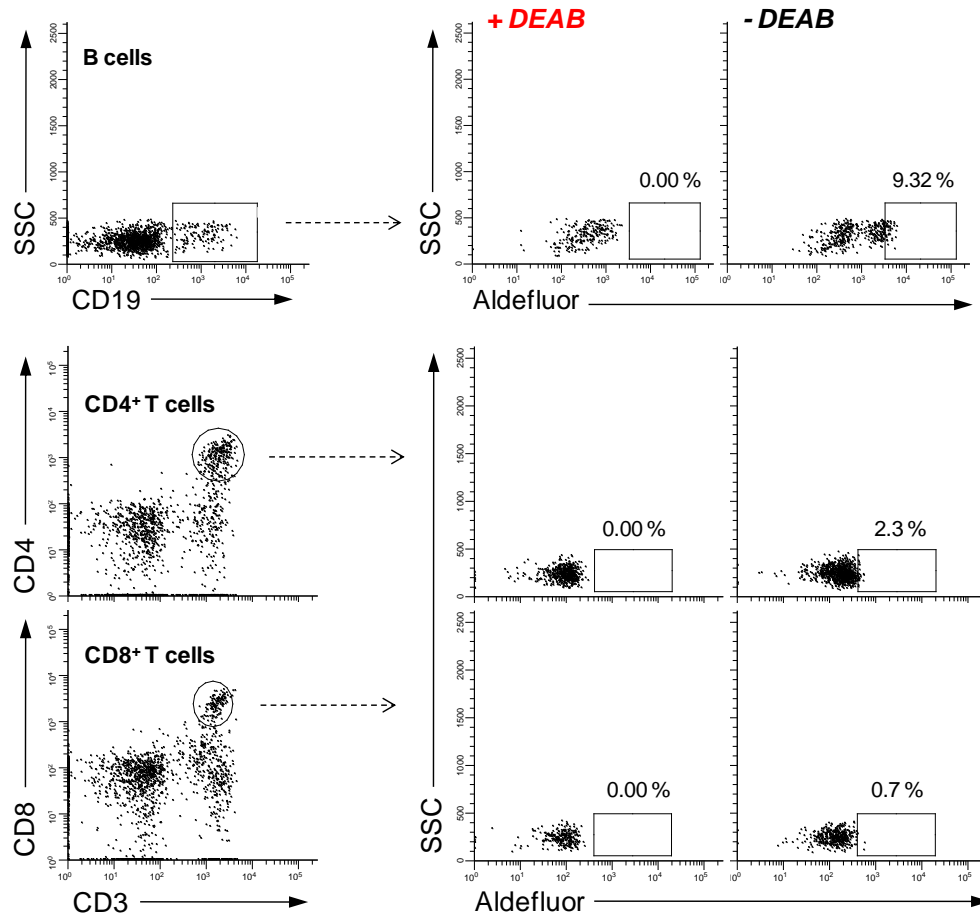


Figure 4.4: Lymphocytes have low levels of RALDH activity. Aldefluor assay was performed in conjunction with flow cytometry to identify RALDH activity in human B cells (CD19⁺ cells), and CD4⁺ and CD8⁺ T cells in LPCs extracted from collagenase-digested healthy control colonic biopsies. Aldefluor⁺ gating was defined in comparison to a DEAB-inhibited control reaction (left column); a fixed separation was introduced between DEAB inhibited cells and the gate to ensure only cells with enhanced RALDH activity were analysed; SSC is side-scatter.

4.4). Approximately 9 % of these cells were Aldefluor⁺, approximately half the level observed in the APC subsets studied. This activity has not previously been identified and may be linked to RA-mediated IgA production as observed in mice (Mora et al., 2006). Very low level levels of Aldefluor/RALDH activity were observed in CD4⁺ and CD8⁺ T cells (2.3 and 0.7 %, respectively). Overall these data support a more general

role for RALDH activity in human intestinal myeloid APCs than the specific functions identified in murine CD103⁺ DCs.

Multiple RALDH isoforms have been identified and could be responsible for the Aldefluor activity observed in human intestinal APCs. In order to define the specific enzymes responsible, human intestinal DCs and CD14⁺ MΦ were sorted by flow cytometry for analysis of *ALDH1A* expression by real-time PCR. The low numbers of DCs obtained using this approach presented a significant technical challenge, and no *ALDH1A* isoforms could be detected in CD103⁺ DCs from a healthy control mucosal sample based on approximately 800 sorted cells. Expression of *ALDH1A2* and *ALDH1A3* was however detected in approximately 8500 CD103⁻ DCs from this sample, whilst *ALDH1A1* was not detected (Figure 4.5). This is consistent with RALDH2 and RALDH3 as responsible for the observed Aldefluor activity of CD103⁻ DCs, and potentially also of CD103⁺ DCs. In contrast to CD103⁻ DCs, CD14⁺ MΦ expressed significant levels of *ALDH1A1*, as well as both *ALDH1A2* and *ALDH1A3* at levels comparable with CD103⁻ DCs (Figure 4.5). A similar expression profile was observed in

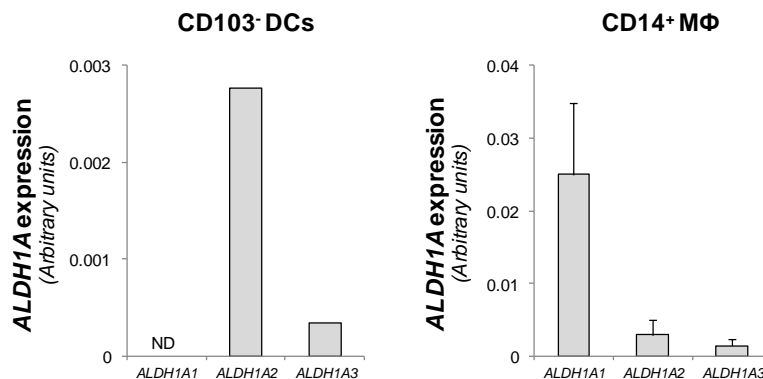


Figure 4.5: Human intestinal CD103⁻ DCs and CD14⁺ MΦ have different *ALDH1A* expression profiles. CD103⁻ DCs and CD14⁺ MΦ were sorted from collagenase-digested healthy control colonic resection tissue or colonic biopsies (CD14⁺ MΦ only) via flow cytometry as Lin⁻ HLA-DR⁺ (n=1) and CD33⁺ CD14⁺ cells (n=7), respectively; *ALDH1A* expression determined by real-time PCR. *ALDH1A* expression normalized to *GAPDH* (DCs) or *GAPDH/RPL30* (CD14⁺ MΦ); mean value displayed + SD; ND is not detected.

CD14⁺ MΦ across 7 independent samples, sorted as CD14⁺ CD33⁺ cells; the superior numbers of CD14⁺ MΦ facilitating sorting and analysis by real-time PCR. Overall, these data suggest that distinct profiles of RALDH enzymes drive the observed Aldefluor staining in CD14⁺ MΦ and DCs in human intestinal mucosa.

The overall ability of a cell to produce RA is determined both by the oxidation of retinal to RA by RALDH enzyme activity, and the prior oxidation of retinol to retinal by the microsomal short chain dehydrogenase/reductase (SDR) family of enzymes (reviewed by Napoli, 2012). Expression of the retinol dehydrogenase *RDH10* was detected in CD103⁺ and CD103⁻ DCs (Figure 4.6). This expression was approximately 4.5 fold higher in CD103⁺ compared with CD103⁻ DCs in intestinal mucosa from a healthy control patient, and is particularly significant considering the low numbers of CD103⁺ DCs obtained through sorting. Furthermore, *RDH10* expression was also identified in CD14⁺ MΦ (see section 4:4.3, Figure 4.14B). This indicates that all three subsets possess the complete enzymatic machinery to convert retinol to RA, and that CD103⁺ DCs may possess an enhanced ability to generate retinal via RDH10 compared with their CD103⁻ counterparts, perhaps resulting in increased overall RA output.

Having identified the presence of RALDH activity in human intestinal DCs and CD14⁺ MΦ, the next step was to establish whether this activity was sufficient to influence cell

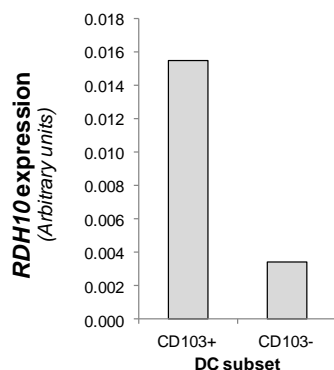


Figure 4.6: Human intestinal DCs express *RDH10*. CD103⁺ and CD103⁻ DCs were sorted by flow cytometry from collagenase-digested healthy control colonic resection tissue and *RDH10* expression determined by real-time PCR. *RDH10* expression normalized to *GAPDH*.

function. In order to assess this, DCs were sorted as Lin⁻HLA-DR⁺ cells via flow cytometry and used to stimulate CFSE-labelled allogeneic naive CD4⁺ T cells. Naive CD4⁺ T cells alone did not proliferate, remaining CFSE^{hi}. In contrast, a significant proportion of CD4⁺ T cells did proliferate in the presence of sorted DCs and became CFSE^{lo} (Figure 4.7A). Proliferating cells expressed high levels of $\alpha_4\beta_7$, consistent with the provision of RA during T cell activation by the DCs. Due to the low numbers of sorted DCs, it was not possible to run multiple co-cultures in which RA signalling was blocked. A separate experiment was therefore performed in which naive CD4⁺ T cells were stimulated with 'walk-out' cells which are enriched for mature DCs (Bell et al., 2001) in the presence or absence of the RAR α antagonist Ro41-5253 (Apfel et al., 1992). Walk out cells induced strong proliferation in naive CD4⁺ T cells as seen by CFSE dilution and high levels of $\alpha_4\beta_7$ expression as with sorted DCs (Figure 4.7B). In the presence of Ro41-5253 the level of $\alpha_4\beta_7$ expression on proliferating T cells was significantly reduced (Figures 4.7B and C), strongly indicating that provision of RA during T cell activation is responsible for the induction of $\alpha_4\beta_7$ expression in human T cells as seen in the mouse (Iwata et al., 2004). These data support the conclusion that the RALDH activity observed in human intestinal DCs generates sufficient RA to allow the efficient induction of a gut-homing phenotype on naive CD4⁺ T cells.

4:4.2 RALDH activity is not reduced in human intestinal CD103⁺ DCs in IBD

Having established the presence of RALDH activity in human intestinal CD103⁺ and CD103⁻ DCs and also CD14⁺ M Φ , the next stage was to determine if the level of this activity was modified in mucosa derived from IBD patients. To address this, the Aldefluor and flow cytometry analysis as described previously in this chapter were

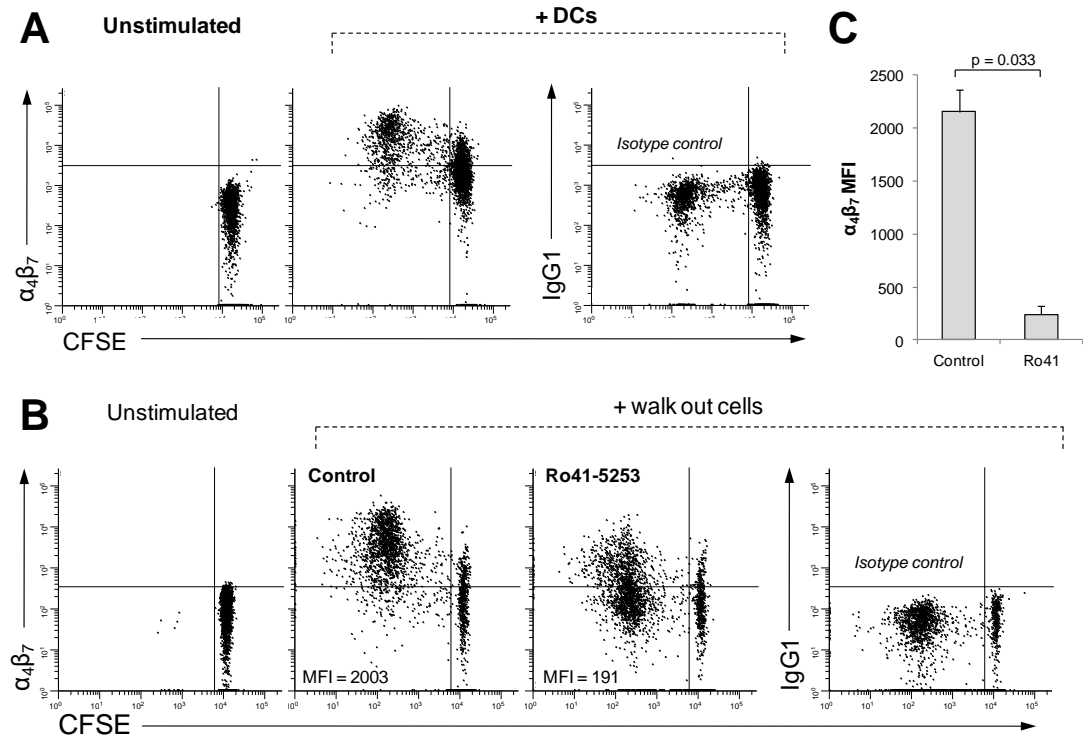


Figure 4.7: Human intestinal DCs induce RA-dependent $\alpha_4\beta_7$ expression on naive CD4⁺ T cells. **A:** DCs were sorted via flow cytometry as Lin⁻ HLA-DR⁺ cells from LPCs derived from collagenase-digested resected human intestinal mucosa. Following 5 days co-culture with CFSE-labelled allogeneic naive CD4⁺ T cells, $\alpha_4\beta_7$ expression was detected on proliferating CFSE^{lo} CD4⁺ T cells via flow cytometry. One example of two independent experiments shown; left panel is T cells alone (unstimulated), right panel indicates isotype control staining. **B:** CFSE-labelled naive CD4⁺ T cells were stimulated with ‘walk out’ cells derived from human intestinal biopsies in the presence or absence of the RAR α antagonist Ro41-5253. MFI of $\alpha_4\beta_7$ on CFSE^{lo} cells is depicted. **C:** Bar chart depicts mean $\alpha_4\beta_7$ MFI + SD from two independent ‘walk out’ co-cultures from the same patient; paired t-test.

applied to LPCs derived from IBD patients undergoing colonic and/or ileal surgical resection due to severe inflammation that had resisted therapy. This included patients with CD in which either the colon or terminal ileum were affected as well UC in which only the colon was affected. As with healthy controls, CD103⁺ and CD103⁻ DCs as well as CD14⁺ M Φ possessed cells with detectable Aldefluor/RALDH activity (Figure 4.8).

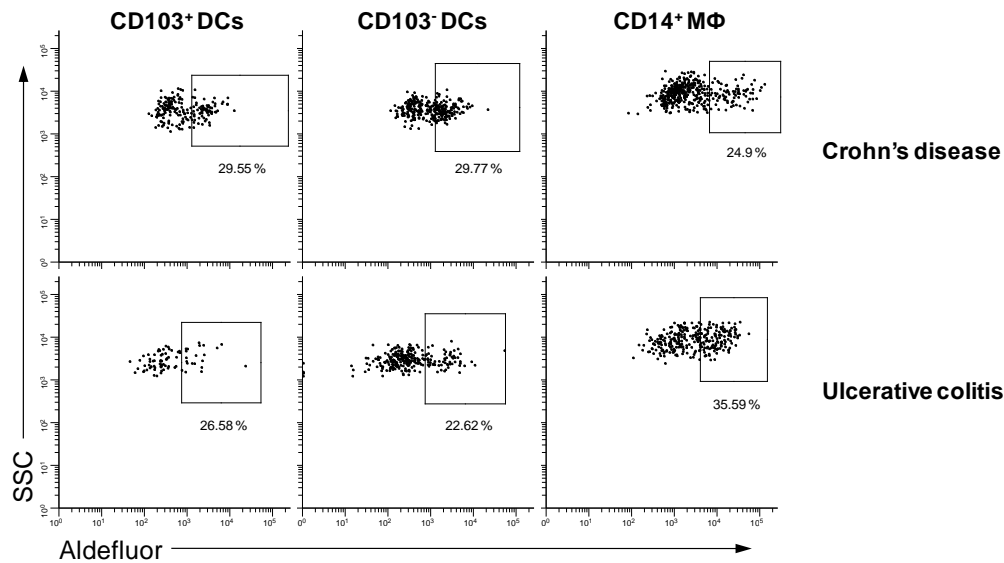


Figure 4.8: RALDH activity is identified in all APC subsets studied in both CD and UC mucosa. Aldefluor assay was performed in conjunction with flow cytometry to identify CD103⁺ and CD103⁻ DCs and CD14⁺ MΦ with enhanced RALDH activity from IBD colon and/or ileal surgical resections. Aldefluor⁺ gating was defined in comparison to a DEAB-inhibited control reaction (not shown); a fixed separation was introduced between DEAB inhibited cells and the gate to ensure only cells with enhanced RALDH activity were enumerated. Both examples above derive from inflamed mucosa.

A direct comparison was then made of the proportion of cells within each subset with detectable Aldefluor/RALDH activity in healthy control and IBD patients, which were separated into 'inflamed' and 'non-inflamed' samples. Of note, 'non-inflamed' in this context refers to macroscopically normal mucosa where inflammation was present elsewhere in the colon or terminal ileum of the patient. In contrast to two murine models of IBD (Collins et al., 2011, Laffont et al., 2010), reduced RALDH activity in human intestinal CD103⁺ DCs in IBD was not observed in this study (Figure 4.9A). Furthermore, the average proportion of Aldefluor⁺ CD103⁺ DCs was increased in both non-inflamed and inflamed IBD compared with healthy control mucosa (27.9, 27.1 and 18.4 %, respectively), although this difference did not reach statistical significance. The trend was more apparent in CD rather than UC samples due to the greater number of

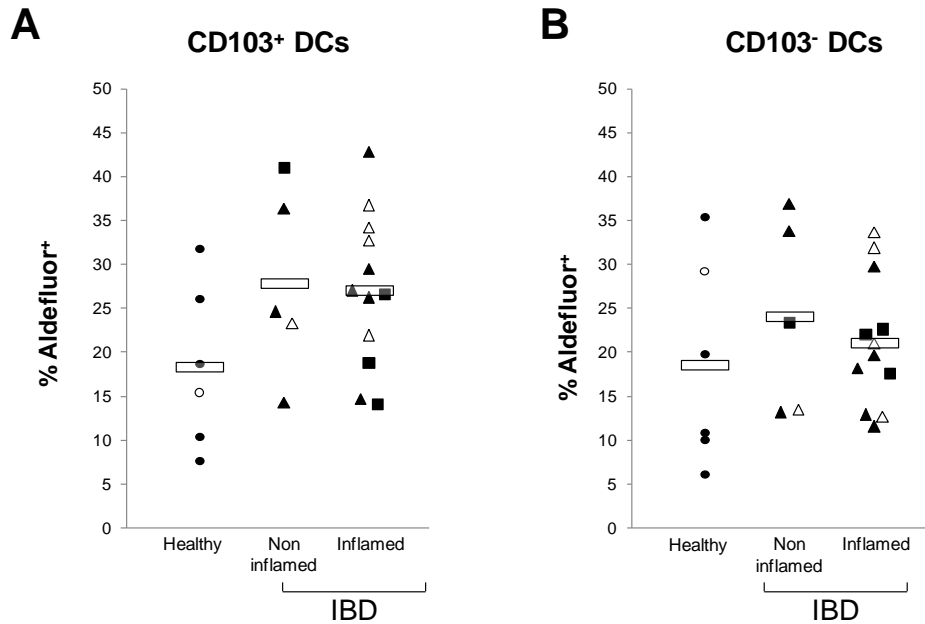


Figure 4.9: Human intestinal CD103⁺ DCs have enhanced RALDH activity in IBD. **A:** Aldefluor and flow cytometry analysis were performed on healthy control (n=6) and non-inflamed (n=5) and inflamed (n=12) IBD LPCs as described previously in this chapter; collated results are shown for CD103⁺ DCs. **B:** Collated results shown for CD103⁻ DCs. ● = Healthy ▲ = CD, ■ = UC; filled symbols are colonic and empty symbols are ileal tissue samples; bars represent mean values.

CD samples obtained (13 versus 4). Of the 3 inflamed UC samples, none displayed enhanced Aldefluor/RALDH activity in CD103⁺ DCs. The single non-inflamed UC sample did however possess high levels of Aldefluor/RALDH activity in CD103⁺ DCs. These data therefore support the conclusion that RALDH activity is maintained or possibly enhanced in human intestinal CD103⁺ DCs in IBD, particularly in CD. Increased activity is also observed in non-inflamed areas which may indicate that RALDH activity becomes enhanced prior to the onset of overt inflammation.

As with CD103⁺ DCs, human intestinal CD103⁻ DCs also did not display reduced Aldefluor/RALDH activity in IBD (Figure 4.9B). The proportion of Aldefluor⁺ CD103⁻ DCs was however similar between non-inflamed and inflamed IBD and healthy control mucosa (24.1, 21.1 and 18.6 %, respectively) with no indication of an enhancement in

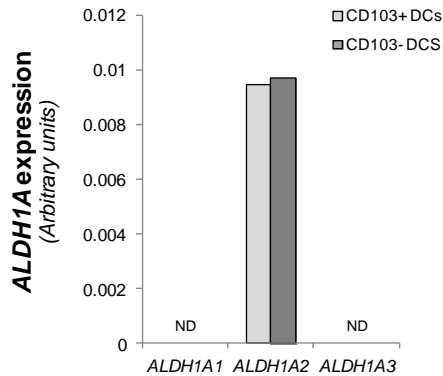


Figure 4.10: *ALDH1A2* expression is detected in intestinal CD103⁺ and CD103⁻ DCs in IBD. DC subsets were sorted via flow cytometry from LPCs derived from surgically resected inflamed CD ileum and *ALDH1A* expression was determined by real-time PCR. Expression normalised to *GAPDH*; ND is not detected.

IBD samples. This may indicate differential regulation of RALDH activity in human intestinal CD103⁺ and CD103⁻ DCs in health and IBD.

LPCs derived from an inflamed ileal CD surgical resection were sorted for CD103⁺ and CD103⁻ DCs by flow cytometry in order confirm *ALDH1A* expression in these subsets in IBD. *ALDH1A2* expression was observed by real-time PCR in both CD103⁺ and CD103⁻ DCs derived from this sample (Figure 4.10). Expression of *ALDH1A1* and *ALDH1A3* were however not detected in either subset. This may indicate that the increased RALDH activity of CD103⁺ DCs in IBD is due to increased expression or alternatively modified post-transcriptional regulation of *ALDH1A2*. Furthermore, *RDH10* expression was again enhanced approximately 4 fold in CD103⁺ compared with CD103⁻ DCs (Figure 4.11); indicating that enhanced conversion of retinol to retinal by CD103⁺ DCs is preserved in IBD. However further samples would be required to confirm this observation.

The proportion of CD103⁺ DCs relative to other APC subsets is significantly reduced in the inflamed murine intestine (Collins et al., 2012, Laffont et al., 2010). A reduced proportion of human CD103⁺ DCs has also been recently observed in our laboratory in an analysis of non-inflamed colonic tissue from CD patients (McCarthy et al., *manuscript in preparation*). A similar trend was observed in the current study with

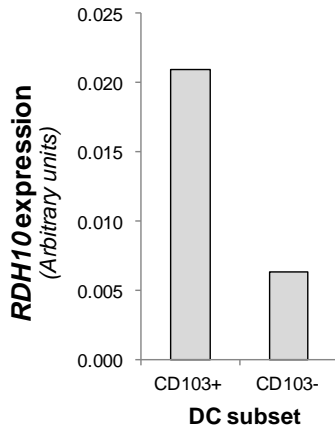


Figure 4.11: Human intestinal CD103⁺ DCs have enhanced *RDH10* expression in IBD. CD103⁺ and CD103⁻ DCs were sorted from collagenase-digested inflamed ileum derived from a CD patient via flow cytometry. *RDH10* expression determined by real-time PCR; expression normalized to *GAPDH*.

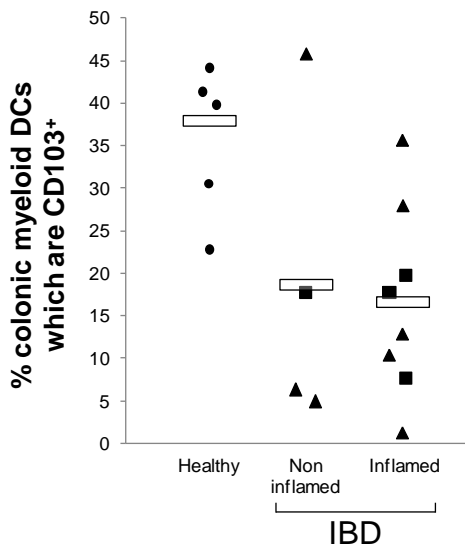


Figure 4.12: The proportion of human colonic CD11c⁺ DCs that are CD103⁺ is reduced in IBD. Data derived from cells as in Figure 4.9, proportion of CD45⁺ Lin⁻ HLA-DR⁺ CD11c⁺ DCs that are CD103⁺ is shown. ● = Healthy, ▲ = CD, ■ = UC; filled symbols are colonic and empty symbols are terminal ileal tissue samples; bars represent mean values.

relatively fewer colonic CD103⁺ DCs in IBD patients as a proportion of total myeloid DCs (Figure 4.12). This trend was not observed with CD103⁺ DCs from the TI of IBD patients however (data not shown).

4:4.3 RALDH activity is significantly enhanced in human intestinal CD14⁺ MΦ in IBD

A direct comparison was made of RALDH activity in intestinal CD14⁺ MΦ in healthy control and IBD patients. The proportion of Aldefluor⁺ CD14⁺ MΦ was significantly enhanced in LPCs derived from both non-inflamed ($p = 0.008$) and inflamed IBD ($p =$

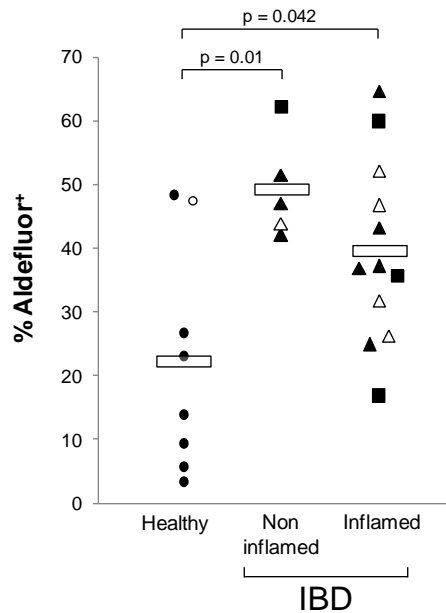


Figure 4.13: Human intestinal CD14⁺ MΦ have significantly enhanced RALDH activity in IBD.

A: Aldefluor and flow cytometry analysis was performed on healthy control and IBD LPCs as described previously in this Chapter; collated results are shown for CD14⁺ MΦ identified as Lin⁺ HLA-DR⁺ cells. ● = Healthy, ▲ = CD, ■ = UC; filled symbols are colonic and empty symbols are terminal ileal tissue samples; bars represent mean values. P-values derived from one way ANOVA with Tukey test.

0.032) compared with healthy control mucosa (49.3, 39.7 and 22.3 %, respectively; Figure 4.13), consistent with enhanced RALDH activity. Due to the superior number of samples, this relationship was again more apparent in CD than UC, but clearly enhanced activity could be seen with both diseases. The proportion of Aldefluor⁺ CD14⁺ MΦ was slightly higher and displayed a reduced degree of variation in non-inflamed compared with inflamed mucosa. The increased proportion of Aldefluor CD14⁺ MΦ in both non-inflamed and inflamed colonic mucosa of IBD patients compared with healthy control mucosa was statistically significant in the absence of data from CD14⁺ MΦ of the TI, with P-values of 0.007 and 0.026, respectively (data not shown).

In order to determine if modified *ALDH1A* expression was responsible for the increased Aldefluor/RALDH activity of CD14⁺ MΦ in IBD, these cells were analysed by real time PCR following sorting by flow cytometry. There was a statistically significant ($p = 0.035$) increase in *ALDH1A1* expression in CD14⁺ MΦ from inflamed intestinal mucosa of CD patients compared with healthy controls (Figure 4.14A). In contrast, expression of *ALDH1A2* and *ALDH1A3* in CD14⁺ MΦ was not significantly different between

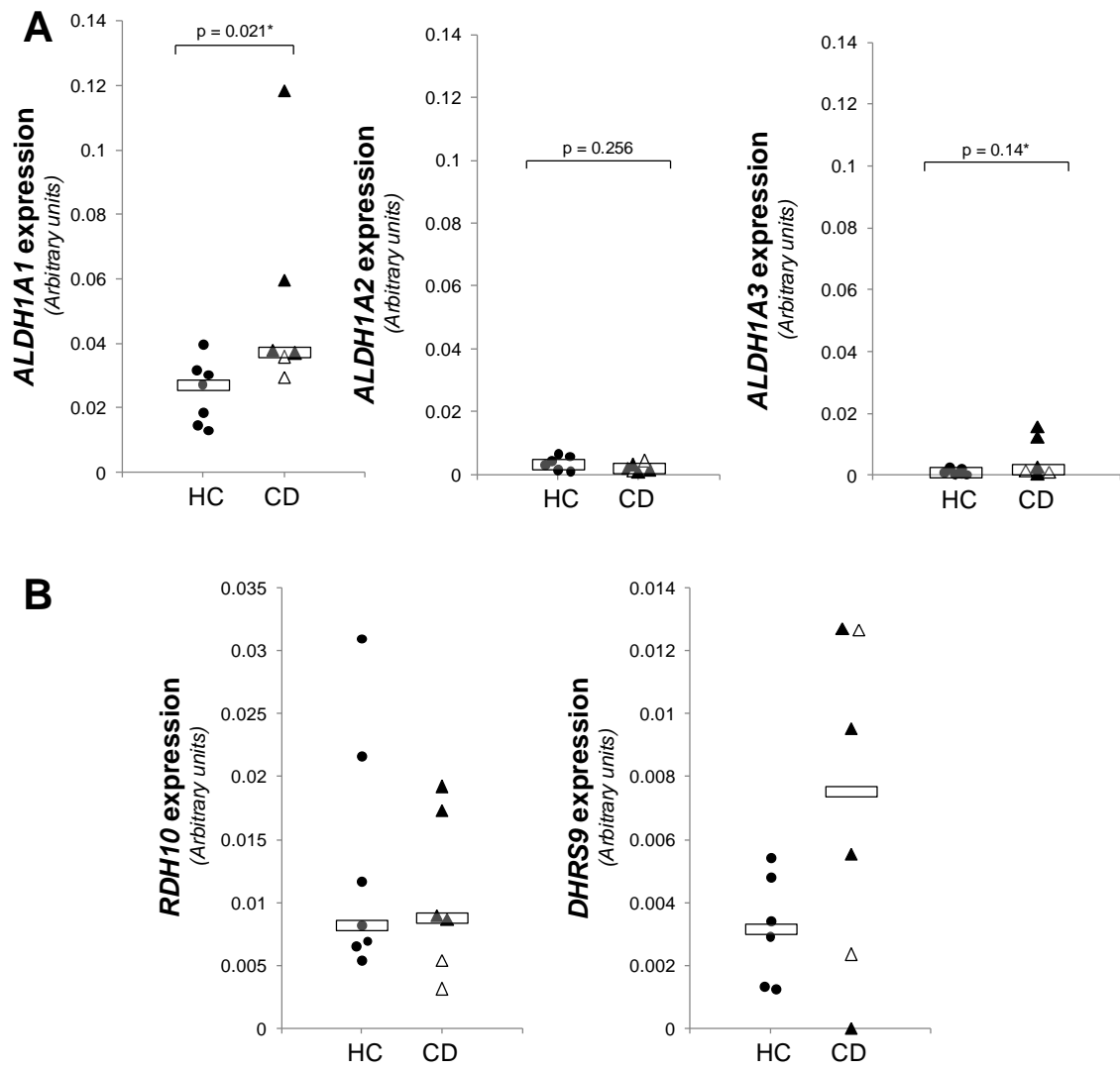


Figure 4.14: *ALDH1A1* expression is enhanced in human intestinal CD14⁺ MΦ in CD.

A: CD14⁺ MΦ were sorted by flow cytometry as CD33⁺ CD14⁺ cells from healthy control and/or inflamed ileal surgical resection or endoscopic biopsies from CD patients. *ALDH1A* isoform expression was determined by real-time PCR; expression normalised to *GAPDH* and *RPL30*. **B:** Expression of *RDH10* and *DHRS9* were determined in the same samples by real-time PCR; expression normalised to *GAPDH* and *RPL30*. ● = Healthy, ▲ = CD; filled symbols are colonic and empty symbols are ileal tissue samples; bars represent median values except for *ALDH1A2* (mean); P-values derive from t-tests; * indicates p-value following log₁₀ transformation of non-normally distributed data.

inflamed CD mucosa and healthy controls. This indicates that enhanced transcription of *ALDH1A1* leads to enhanced RALDH1 activity, contributing to the observed increase in Aldefluor activity of CD14⁺ MΦ in CD.

In contrast with *ALDH1A1*, no statistically significant differences were observed in the levels of *RDH10* or *DHRS9* expression in CD14⁺ MΦ in inflamed CD and healthy control intestinal mucosa (Figure 4.14B) although samples sizes were small. This indicates that the ability to oxidise retinol to retinal, at least via RDH10 and DHRS9, is unchanged in CD14⁺ MΦ in IBD but that conversion of retinal to RA is potentially increased.

4:4.4 RALDH activity is present in non-haematopoietic cells and is reduced in IBD

Non-haematopoietic cells were excluded from analysis of RALDH activity in APCs by gating on CD45⁺ cells. Significant Aldefluor staining was however observed within the CD45⁻ compartment (Figure 4.15A), indicating that non-haematopoietic cells are an additional source of RALDH activity within the human intestinal mucosa. The EDTA incubation step was omitted when processing biopsies to assess this activity so as to avoid the loss of epithelial cells, which were a potential source of the activity. The level of Aldefluor staining in colonic CD45⁻ cells was reduced in CD (Figures 4.15B and C), and also in a single UC sample derived from a non-inflamed biopsy. This indicates that in contrast to human intestinal myeloid APCs, non-haematopoietic cell RALDH activity is reduced in IBD.

Variable expression of HLA-DR was detected on CD45⁻ cells, and expression was typically enhanced on cells derived from IBD biopsies (Figures 4.15D and E).

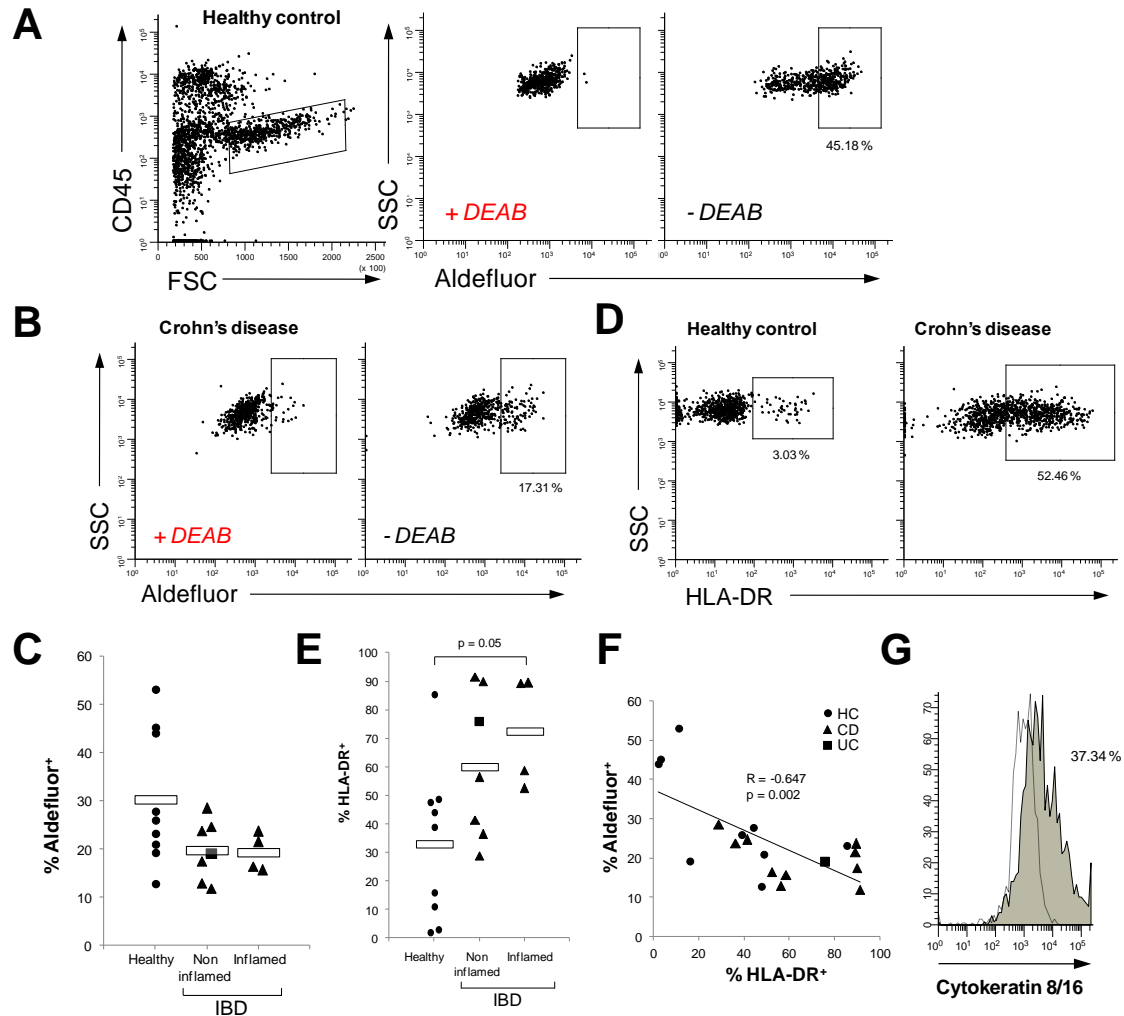


Figure 4.15: Human colonic CD45⁺ cells have RALDH activity which is reduced in IBD and negatively correlates with HLA-DR expression. **A:** Aldefluor analysis was performed on LPCs following collagenase digestion of colonic biopsies; analysis was focussed on CD45⁺ cells, in comparison to a DEAB inhibited control as previously. **B:** Aldefluor analysis was performed on CD45⁺ cells derived from IBD biopsies in addition to healthy controls; example from a CD patient shown. **C:** Collated CD45⁺ Aldefluor data. **D:** HLA-DR expression was detected on CD45⁺ cells, and was increased in cells derived from IBD mucosa. **E:** Collated HLA-DR expression on CD45⁺ cells from healthy control and IBD biopsies. P-value derives from ANOVA with Tukey test. **F:** HLA-DR expression negatively correlates with proportion of Aldefluor⁺ CD45⁺ cells. P-value derived from a Spearman's rank test, coefficient of correlation indicated as R. **G:** A significant proportion of CD45⁺ cells are epithelial: cytokeratin 8/16 measured following permeabilisation of LPCs; filled histogram represents antibody staining, dotted histogram represents the relevant isotype control. ● = Healthy, ▲ = CD, ■ = UC; bars represent mean values.

Furthermore, a significant negative correlation was observed between the proportion of HLA-DR expressing cells and overall RALDH activity within the CD45⁺ compartment (Figure 4.15F). A significant proportion of the CD45⁺ cells expressed cytokeratin 8 and/or 16, indicating the presence of intestinal epithelial cells (Moll et al., 1982). This indicates that the observed Aldefluor/RALDH activity may at least in part derive from colonic epithelial cells. HLA-DR expression is induced on these cells by inflammatory cytokines (Pallone et al., 1988), and so overall these data support the conclusion that RALDH activity in non haematopoietic cells including epithelial cells is negatively regulated by inflammatory signals in IBD.

4:4.5 Total tissue RA levels are unchanged in IBD compared with healthy intestinal mucosa

Having observed enhanced RALDH activity in CD14⁺ M Φ and CD103⁺ DCs and reduced activity in non-haematopoietic cells in IBD, the next step was to determine the impact of these changes on overall tissue RA levels. To address this, intestinal biopsies were snap frozen in liquid nitrogen and shipped for analysis to the lab of Maureen Kane at the University of Maryland for quantification of retinoids by liquid chromatography with tandem mass spectrometry (LC/MS/MS) (Kane et al., 2005). This preliminary analysis did not detect significant differences in the tissue levels of RA in biopsies derived from healthy control and CD patients (Figure 4.16). The average level of RA was approximately 40 pmol/g protein.

In contrast to RA, the levels of retinol were reduced in CD compared with healthy controls, although this difference did not reach statistical significance (Figure 4.16). The average level detected in non-inflamed biopsies was approximately halfway between healthy control and inflamed biopsies (89.5 compared with 123.8 and 69.9 nmol/g

protein, respectively). Furthermore, the storage form of retinol, retinyl ester, was also reduced in inflamed CD compared with healthy control biopsies (41.7 and 57.6 nmol/g protein), with only a small difference between inflamed and non-inflamed CD biopsies.

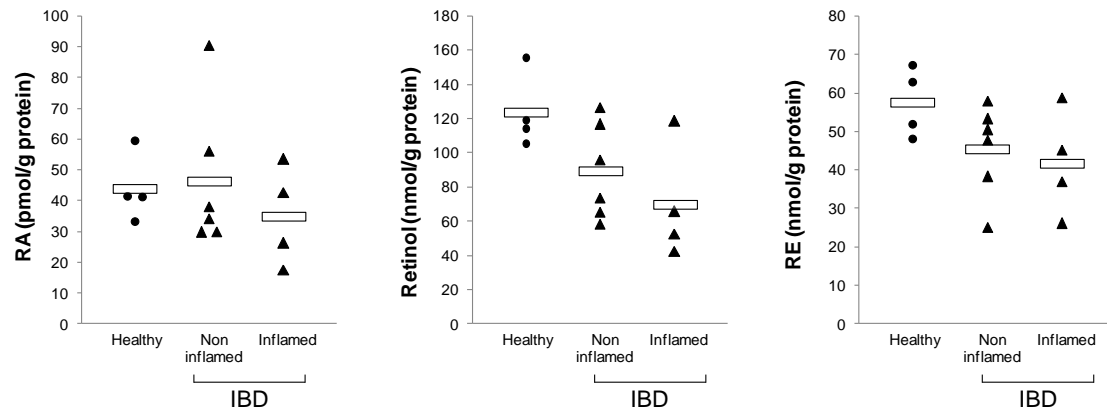


Figure 4.16: RA levels are unchanged whilst retinol levels are reduced in colonic biopsies in IBD. Colonic biopsies were snap frozen in liquid nitrogen and analysed by HPLC-MS/MS (Kane et al., 2005). Retinoids are quantified in each case relative to the mass of protein present within the biopsies. Bars indicate mean values; ● = healthy, ▲ = CD.

4:5 Discussion

In this chapter, RALDH activity in human intestinal APCs was characterised using the Aldefluor assay as described in Chapter 3. Focussing on both the colon and TI, RALDH activity was identified in CD103⁺ and CD103⁻ DCs, as well as in CD14⁺ MΦ. Activity in these subsets varied between individuals, but no single subset displayed enhanced levels of activity. RALDH activity was not common to all haematopoietic cells however as T cells did not stain in the Aldefluor assay. A small population of B cells stained weakly with the Aldefluor reagent but the significance of this low level of potential RALDH activity is unclear.

In the mouse, RALDH activity is particularly enhanced in CD103⁺ DCs found in the SI-LP, particularly the duodenum and jejunum (Schulz et al., 2009, Denning et al., 2011, Jaensson-Gyllenback et al., 2011), as well as the MLN (Coombes et al., 2007, Yokota et al., 2009, Molenaar et al., 2011, Guillems et al., 2010) which reflects migration of SI-derived DCs (Johansson-Lindbom et al., 2005, Jaensson et al., 2008). Murine colonic and TI CD103⁺ DCs however have lower levels of RALDH activity (Denning et al., 2011, Jaensson-Gyllenback et al., 2011, Villablanca et al., 2011b). The equivalent RALDH activity observed in CD103⁺ and CD103⁻ DCs in this study may be consistent with the murine data if the level of activity in these cells is considered basal for myeloid cells in the colon and TI LP.

Enhanced RALDH activity is inferred in human MLN CD103⁺ DCs based on a specialised ability to imprint naive CD8⁺ T cells with CCR9 expression (Jaensson et al., 2008). It was not possible to confirm the presence of enhanced RALDH activity in these cells as MLN were not available in this study. However, future work in which human duodenal or jejunal mucosal CD103⁺ DCs are characterised would confirm whether

RALDH activity is significantly enhanced in human proximal SI-LP. It remains possible that RALDH levels in all cell populations examined in the study could be lower than those of cells in the proximal small intestine.

Real-time PCR indicated that *ALDH1A2* expression was responsible for the RALDH activity observed in CD103⁻ DCs in the healthy human intestine. This is also likely to be the case for CD103⁺ DCs, as *ALDH1A2* was identified in these cells in a sample derived from a CD patient. This is consistent with the mouse in which *Aldh1a2* expression is identified in MLN (Coombes et al., 2007) and SI-LP CD103⁺ DCs (Schulz et al., 2009, Yokota et al., 2009). *ALDH1A1* expression was not identified in CD103⁺ or CD103⁻ DCs in this study, although may have been present below the limit of detection. In the mouse, low levels of *Aldh1a1* expression have been observed in SI-LP CD103⁺ CD11b⁺ DCs and lower levels in CD103⁺ CD11b⁻ DCs (Denning et al., 2011). *Aldh1a1* was also detected in PP DCs in mice (Iwata et al., 2004), although the gating strategy used (MHCII⁺ CD11c⁺) is not sufficient to have excluded macrophages (Schulz et al., 2009).

In contrast with DCs, *ALDH1A1* expression was routinely detected at high levels in CD14⁺ MΦ in this study. This is may be the result of upregulation within the intestinal mucosa or could be intrinsic to this lineage of cells. Expression was approximately 9 fold higher than *ALDH1A2*, whilst *ALDH1A3* was also present. *ALDH1A* expression in CD14⁺ MΦ in this study is consistent with their observed RALDH activity. The relative contributions of the separate *ALDH1A* isoforms to the overall RALDH activity of CD14⁺ MΦ is unclear at present, although *ALDH1A1* is expressed at sufficiently high levels to drive at least some of this activity. Interestingly, high levels of *Aldh1a1* expression are found in murine CD11c⁻ F4/80⁺ MΦ in the SI-LP (Denning et al., 2011). Elevated *ALDH1A1/Aldh1a1* expression may therefore be common to human and murine

monocyte-derived cells. This idea is developed further in Chapter 5, where *ALDH1A1* expression is also identified in human CD14⁺ blood monocytes.

This significance of multiple RALDH isoforms expressed simultaneously within CD14⁺ MΦ, and why *ALDH1A1* expression is not observed in intestinal DCs is unclear. One hypothesis is that different RALDH enzymes exist in specific locations within cells, and generate distinct intracellular pools of RA (Napoli, 2012). This notion is supported by the distribution of RALDH enzymes in rat primary hippocampus astrocytes. RALDH1 is located both in the cytosol but also a significant quantity is observed in the nucleus of these cells. In contrast, RALDH2 is concentrated within a perinuclear region (Wang et al., 2011a). Furthermore, RALDH1, 2 and 3 all make distinct contributions to the overall RA output of astrocytes (Wang et al., 2011a). RALDH1, 2 and 3 are also differentially expressed in different organs during pre-natal development of mice, indicating that they are each involved in distinct contexts where RA generation is required (Niederreither et al., 2002). The distribution of RALDH enzymes in human intestinal CD14⁺ MΦ and DCs is unknown and could be studied further by immunohistochemistry.

The substrate specificities of the RALDH enzymes are also distinct. Human RALDH1 oxidises *all-trans*, *13-cis* and *9-cis* retinal with equal efficiency (Bhat and Samaha, 1999), whilst murine RALDH2 oxidises *all-trans* retinal with greater efficiency than the other isoforms (Gagnon et al., 2002). In contrast, murine RALDH3 catalyses only *all-trans* retinal with approximately 10 fold greater efficiency than RALDH1 and RALDH2 (Sima et al., 2009). Primary rat astrocytes express all three RALDH enzymes, and each contributes to the net output of RA (Wang et al., 2011a). RALDH enzymes are also differentially regulated by retinoids. RALDH1 in rats is inhibited by cellular retinol binding protein (CRBP)-I which is not in association with retinol (apo-CRBP-I),

indicating low levels of cellular retinol (Posch et al., 1992). In contrast, RALDH2 in rats is not inhibited by apo-CRBP-I (Zhai et al., 2001).

The ability to produce RA is widely inferred from expression of RALDH enzymes. However data in Chapter 6 working with MoDCs suggest that RALDH activity is not always indicative of release of functional RA. Therefore, it was important to seek further evidence to support RA production by APC populations isolated from intestinal tissue. Small cell numbers precluded direct RA measurement therefore an indirect approach was adopted. DCs sorted via flow cytometry as Lin⁻ HLA-DR⁺ cells were able to induce $\alpha_4\beta_7$ expression on allogeneic naive CD4⁺ T cells in this study. Furthermore, in 'walk out' T cell stimulation cultures induction of $\alpha_4\beta_7$ was shown to be RA-dependent. These data indicate that the RALDH activity observed in DCs in this study generates sufficient RA for the induction of this 'gut-homing' phenotype. An alternative explanation for the induction of $\alpha_4\beta_7$ is that DCs pick up RA from the intestinal mucosa and simply transfer it to T cells during activation. This is particularly important as only low concentrations (0.1 nM in mice) of RA are required to induce $\alpha_4\beta_7$ expression (Iwata et al., 2004). This possibility has been addressed directly in the context of murine DCs (Jaensson-Gyllenback et al., 2011). MLN DCs in this study were unable to induce CCR9 on CD8⁺ T cells where retinol had been removed from the growth medium, either by use of a serum-free media (X-Vivo10) or else destruction of retinoids in the media by UV-irradiation of FCS. The authors concluded that metabolism of retinol to RA by the DCs and not simple carryover of RA was required to induce CCR9 expression. Whilst this does not confirm that the $\alpha_4\beta_7$ expression induced by sorted intestinal DCs in this study is the result of retinol metabolism specifically within DCs, it does provide significant supporting evidence. These experiments focussed exclusively on $\alpha_4\beta_7$ expression as CCR9 expression was routinely very low or absent on CD4⁺ T cells following 'walk out'

stimulation cultures in this study, and even during activation of naive CD4⁺ T cells in the presence of exogenous RA (data not shown).

Based on a limited number of samples, expression of *RDH10* was approximately 4 fold enhanced in CD103⁺ compared with CD103⁻ DCs in this study. RDH10 is a microsomal short chain dehydrogenase/reductase (SDR) which exerts significant influence over the rate of RA production by catalysing the oxidation of retinol to retinal which is rate-limiting in the presence of CRBP-I (Napoli, 1986, Wang et al., 2011a). Whether this level of enhancement is sufficient to affect the function of CD103⁺ DCs is unclear. It may however indicate that CD103⁺ DCs possess a superior ability to oxidise retinol to retinal and so provide a greater quantity of substrate for RALDH enzymes, ultimately resulting in enhanced RA production. The low numbers of CD103⁺ obtained during cell sorting precluded further analysis of the influence of RDH10 on overall RA output. However, this would be an interesting area to develop where sufficient material is available. Further investigation would be required to both confirm and determine the functional significance of this observation.

Reduced RALDH activity has been reported in CD103⁺ DCs in both the TNFΔARE (Collins et al., 2011) and T cell transfer murine models of IBD (Laffont et al., 2010). In this study, RALDH activity in CD103⁺ DCs was not reduced in IBD patients compared with healthy controls. There was instead a trend in which CD103⁺ DCs displayed greater Aldefluor/RALDH activity, although this did not reach statistical significance with the relatively small number of samples it was possible to analyse. The majority of samples studied were CD, but enhanced RALDH activity was also apparent in the UC samples analysed. This was observed in both inflamed and non-inflamed IBD mucosa; although importantly in these macroscopically 'non-inflamed' samples significant inflammation was present elsewhere colon and/or ileum of the patient. This tissue may

therefore exist in a 'pre-inflamed' state - whether enhanced RALDH activity is observed in CD103⁺ DCs in quiescent IBD remains to be determined. This should be addressed directly by studying RALDH activity in CD103⁺ DCs in IBD patients in remission.

The enhanced RALDH activity of CD103⁺ DCs may be the result of increased transcription of *ALDH1A2*, or alternatively post-transcriptional regulation of the *ALDH1A* isoforms already present. As discussed in Chapter 3, RA may boost RALDH activity of human MoDCs through post-transcriptional regulation of *ALDH1A2* and so a similar mechanism could operate in CD103⁺ DCs. Further analysis would be required to understand the mechanism of this increased activity, including expression analysis of a large number of samples and also analysis of RALDH2 protein content via methodologies including Western blot.

The contrasting RALDH activity between human and murine CD103⁺ DCs in health and disease may relate to the tissue origin of the DCs in question. In the mouse, high levels of RALDH activity accumulate within CD103⁺ DCs in the SI-LP, possibly due to the presence of RA within the tissue inducing a positive feedback loop (Villablanca et al., 2011b, Molenaar et al., 2011, Jaensson-Gyllenback et al., 2011). Based on BrdU labelling experiments, murine CD103⁺ DCs are known to turnover relatively quickly within the LP (Jaensson et al., 2008, Schulz et al., 2009, Laffont et al., 2010). Moreover, inflammation enhances CD103⁺ DC maturation and migration to the draining MLN in a CCR7-dependent manner (Schulz et al., 2009). Newly recruited CD103⁺ DCs within the inflamed LP may therefore have insufficient time to accumulate high levels of RALDH before undergoing maturation and migration. Furthermore, TNFΔARE mice have reduced levels of RA within the TI (Collins et al., 2011), which may further reduce RA-mediated induction of RALDH in CD103⁺ DCs in inflammation. As this study focused on more distal areas of the human intestine, it is possible that CD103⁺ DCs

possess only basal levels of RALDH activity common to myeloid cells within this tissue. The onset of inflammation would therefore result in migration of these DCs from the LP, and replacement by DCs with at least similar levels of RALDH activity.

The enhanced RALDH activity observed in this study in CD103⁺ DCs may be related to phenotypic differences in DCs in health and IBD. Enhanced expression of CD40 has been reported on human intestinal myeloid DCs in CD (Hart et al., 2005). Whilst DCs are not fully mature in IBD patients (Bell et al., 2001), this may reflect a slightly advanced state of maturation when compared with healthy control patients. RA signalling through RAR α /RXR has previously been implicated in DC maturation (Geissmann et al., 2003), and so a potential explanation is that RALDH activity in CD103⁺ DCs increases as part of or because of DC maturation. To address this directly, RALDH activity of CD103⁺ DCs could be determined via the Aldefluor assay following collagenase digestion of intestinal tissue, and then again after 24 hours in culture to allow time for maturation. In support of this hypothesis, it was demonstrated in Chapter 3 that Aldefluor/RALDH activity of human MoDCs is largely restricted to cells displaying a mature phenotype. Furthermore, MLN CD103⁺ DCs in TNF Δ ARE mice display slightly enhanced levels of *Aldh1a2* expression compared with WT mice at 4 weeks, which is early in the onset of inflammation (Collins et al., 2011). By 20 weeks, and at the full onset of inflammation, *Aldh1a2* expression in MLN CD103⁺ DCs is significantly reduced. This early enhancement of *Aldh1a2* expression may be consistent with the arrival of DCs in a more mature state.

In contrast with CD103⁺ DCs, enhanced RALDH activity was not as clearly observed in CD103⁻ DCs in IBD in this study. This may reflect heterogeneity within the CD103⁻ DC gate as suggested by the reduced levels of CCR7 expression on these cells following maturation. In this sense, the overall RALDH activity of this subset may be reduced by

the presence of cells that are not *bona fide* DCs when compared with the 'purer' CD103⁺ DC population. Alternatively, RALDH activity could be regulated differently in CD103⁺ and CD103⁻ DCs in the context of intestinal inflammation. Other reports have identified a CD1c⁺ subset of DCs in human intestinal mucosa as predominantly CD103⁻ (Dillon et al., 2010). The extent of overlap between CD1c⁺ and CD103⁻ DCs as defined here, and whether these cells possess functional RALDH activity should be the subject of future study.

Whether the enhanced RALDH activity of CD103⁺ DCs would significantly alter the function of DCs is unclear. If IBD and healthy control CD103⁺ DCs ultimately mature to the same extent then they would likely have equivalent ability to produce RA and so influence naive lymphocytes during activation in lymphoid tissue. If however following maturation DCs from IBD patients still have enhanced RALDH activity, then this would be more likely to influence the functional outcomes of antigen presentation. It has previously been shown that human MLN CD103⁺ DCs from CD patients induce a similar level of CCR9 expression on CD8⁺ T cells compared with healthy controls (Jaensson et al., 2008). This may indicate that overall, the differences in RALDH activity observed in CD103⁺ DCs in this study do not significantly modify the ability of these cells to induce RA-dependent responses. Alternatively, enhanced RALDH activity of CD103⁺ DCs may contribute to an observed increase in the proportion of FOXP3⁺ T cells in the colonic mucosa of CD and UC patients (Wang et al., 2011d).

Reduced proportions of colonic CD103⁺ DCs may be attributed to migration into the draining lymphatics, or may simply be relative to influx of CD103⁻ DCs. In the TNFΔARE model of IBD the proportion of ileal CD103⁺ DCs is reduced during inflammation (Collins et al., 2011). It remains unclear why a similar reduction is not observed in the TI samples used in this study. This may relate to the presence of

lymphoid tissue including PPs in the TI which are less frequent in the large intestine (reviewed by Day and Morson, 2003). The dynamics of DC migration and recruitment may be distinct in such tissue under the influence of inflammation. Overall therefore, the study would be further improved by increasing sample numbers to allow for a more systematic comparison of RALDH activity in CD103⁺ DCs in healthy control and IBD patients with a more defined colonic/TI distinction. Taken further, the various colonic segments (ascending, transverse, descending, sigmoid) could be analysed separately to indicate how RALDH activity is modified along the intestinal tract in greater resolution.

In addition to CD103⁺ DCs, this study identified significantly enhanced RALDH activity in CD14⁺ MΦ in IBD patients compared with healthy controls. This appears to be due at least in part to enhanced expression of *ALDH1A1*. An earlier report identified equivalent *ALDH1A2* expression in CD14⁺ MΦ in healthy control and CD patients (Kamada et al., 2009) which was also observed in this study. However, the Kamada study did not measure *ALDH1A1* expression and so it is unclear to what extent it was increased within their patient group.

The Kamada study also identified significantly reduced expression of *RDH10* in CD14⁺ MΦ in CD patients compared with healthy controls (Kamada et al., 2009). However, the work presented in this thesis identified no statistically significant difference in *RDH10* expression between the two groups. The reason for this discrepancy is unclear, though may relate to the particular purification methods employed. In the study by Kamada et al, CD14⁺ MΦ were enriched from LPMCs with CD14⁺ microbeads before being subjected RNA extraction and real-time PCR. In contrast, in this thesis, CD14⁺ MΦ were sorted by flow cytometry as CD33⁺ CD14⁺ cells which may have given a purer population and so more accurate results for *RDH10* expression.

As with CD103⁺ DCs, the role of RDH10 in determining overall RA output of human intestinal CD14⁺ MΦ has not been properly addressed and warrants further attention. The origins and functional significance of enhanced RALDH activity in human intestinal CD14⁺ MΦ in IBD are addressed in Chapter 5.

This study identified the non-haematopoietic compartment (identified as CD45⁻ cells), including epithelial cells, as an additional source of RALDH activity in the human intestinal mucosa. RA signalling through RAR/RXR has previously been implicated in the maintenance of epithelial tight junctions and contributes to the integrity of the epithelial barrier (Osanai et al., 2007). Furthermore, mice maintained on a vitamin A deficient diet display significant alterations in small intestinal epithelial physiology including shortened villi, atrophy and goblet cell hyperplasia (Cha et al., 2010).

The level of RALDH activity of CD45⁻ cells was reduced in IBD compared with healthy controls in this study. This is consistent with reports that expression of *ALDH1A1* is significantly down regulated in colonic epithelial cells in CD (Iliev et al., 2009). In addition, a statistically significant negative correlation was identified between Aldefluor staining and HLA-DR expression on colonic CD45⁻ cells in this study. It has been previously observed that HLA-DR expression is significantly enhanced on human colonic epithelial cells in active IBD compared with healthy controls (Fais et al., 1987). Furthermore, culturing epithelial cells for 24 hours in the presence of IFN γ in vitro induces significant increases in HLA-DR expression (Pallone et al., 1988). It is likely that a significant fraction of the CD45⁻ Aldefluor/RALDH activity is driven by the epithelial cells identified within this population by cytokeratin staining but this could not be tested directly because the cell permeabilisation required for epithelial identification was not compatible with the Aldefluor assay. It is therefore possible that non-epithelial

stromal cells also contribute to this RALDH activity, with reduced overall activity in the presence of inflammation.

The functional significance of this is currently unclear. It has been suggested that small intestinal epithelial-derived RA plays an important role in conditioning intestinal DCs by inducing RALDH activity in mice (McDonald et al., 2012) as well as inducing regulatory properties including the ability to induce of FOXP3⁺ T_{REG} in humans (Iliev et al., 2009). The observed increase in RALDH activity in CD103⁺ DCs in this study suggests that the loss of RALDH activity in colonic epithelial cells does not impact on human colonic DCs in this way, at least at the level of RALDH activity. Whether or not this is also the case in the small intestine remains to be determined.

Based on increased RALDH activity in CD103⁺ DCs and decreased activity in and CD45⁻ cells, it was interesting to note that the overall tissue levels of RA were equivalent between healthy controls and CD patients. Whilst this is based on a limited number of samples, it contrasts directly with the TNFΔARE mouse model of IBD in which RA levels in the TI are significantly reduced compared with WT mice (Collins et al., 2011). The average level of RA including all samples was approximately 42.4 pmol/g protein, or 2.6 pmol/g tissue. This compares with a value of 5 pmol/g tissue detected in the mouse colon using an identical approach (Villablanca et al., 2011b). Whether this difference in the levels of tissue RA between species is sufficient to influence intestinal immunology is unclear. Reduced levels of tissue RA in humans may even explain why CD103⁺ DCs do not display enhanced RALDH activity in this study.

Whilst RA levels were unchanged, levels of retinol did however appear to be reduced in CD patients. Approximately 44 nmol/g protein or 7 nmol/g tissue of retinol were detected in healthy control biopsies, compared with 1 nmol/g tissue in mice (Villablanca

et al., 2011b). Interestingly, mice and humans have similar levels of serum retinol with 0.81 nmol/ml in mice (Kane et al., 2005) and approximately 1 to 2 nmol/ml in humans (Olmedilla et al., 1997). The mechanism and functional significance of a reduction in retinol in the colonic mucosa of CD patients is unclear. It has been previously demonstrated that serum retinol levels are reduced in active CD and UC patients compared with healthy controls, and was attributed primarily to reduced levels of serum retinol binding protein (Janczewska et al., 1991). This study also showed that serum retinol levels return to normal in inactive disease. Reduced retinol concentration within the colonic mucosa of active CD patients therefore may be the result of reduced circulating levels of retinol. Furthermore, the intermediate level of mucosal retinol may result from elevated serum retinol associated with inactive disease. This highlights a close association between serum and mucosal retinol levels in the large intestinal mucosa. Villus-associated enterocytes in the small intestine display specialized expression of CRBP-II, consistent with a key role in uptake of dietary retinol (Li and Norris, 1996). It would therefore be of interest to determine the relationship between serum and mucosal retinol levels in the small intestine of IBD patients.

Despite a reduced level of retinol in large intestinal mucosa of active CD patients, levels of RA were similar between these two states. This may suggest that retinoid metabolism is shifted within the large intestinal mucosa in CD in order to maintain overall levels of RA. This would therefore suggest that net RALDH activity is increased in the intestinal mucosa in IBD, compensating for reduced levels of serum retinol. RA contributes to immune responses in mice in part as an accessory factor e.g. in promoting inflammation in the presence of IL-15 (DePaolo et al., 2011) or favouring induction of Foxp3⁺ T_{REG} in the presence of TGF- β (Coombes et al., 2007, Sun et al., 2007, Mucida et al., 2007). RA may therefore influence ongoing immune responses

within human intestinal mucosa through supporting the activities of the varying cytokine landscape associated with IBD, without a significant change in overall RA levels between healthy controls and IBD patients.

4:6 Conclusion

In conclusion, this chapter has demonstrated for the first time the presence of RALDH activity in CD103⁺ and CD103⁻ DCs as well as CD14⁺ MΦ in distal human intestinal mucosa. This activity stems from *ALDH1A2* and *ALDH1A3* expression in DCs, but predominantly *ALDH1A1* expression in CD14⁺ MΦ. Furthermore, DCs display an ability to induce high levels of $\alpha_4\beta_7$ expression on naive CD4⁺ T cells, consistent with functional RALDH. Contrary to expectation, RALDH activity is not reduced in CD103⁺ DCs in IBD and displays a slight increase in activity which is not observed in CD103⁻ DCs. The hypothesis that loss of RALDH activity in human intestinal CD103⁺ DCs contributes to the pathogenesis of IBD is therefore not supported. In addition, RALDH activity is significantly enhanced in CD14⁺ MΦ in IBD patients. RALDH activity in the non-haematopoietic compartment, including epithelial cells, correlates negatively with inflammation and is reduced in IBD patients. The overall concentration of RA within the intestinal mucosa is similar in CD and healthy control patients, despite an apparent reduction in mucosa retinol levels in CD. In Chapter 5, attention will turn to the origins and functions of enhanced RALDH activity of CD14⁺ MΦ in the pathogenesis of IBD.

Chapter 5: Analysis of the origins and function of enhanced RALDH activity in human intestinal CD14⁺ MΦ in IBD

5:1 Chapter summary

This chapter focuses on determining the origins and function of enhanced RALDH activity in human intestinal CD14⁺ MΦ in IBD reported in Chapter 4. Analysis of Aldefluor/RALDH activity in CD14⁺ blood monocytes, precursors to CD14⁺ MΦ, indicates that RALDH activity is acquired in this lineage prior to arrival in the intestinal mucosa but may be locally upregulated within the distal intestine of IBD patients. Furthermore, enhanced expression of *MN1* suggests that differentiation of CD14⁺ monocytes is altered in IBD; blood monocytes in patients may exist in an earlier state of differentiation compared with their healthy control counterparts. An *in vitro* system of monocyte differentiation indicates that RA plays an important role in the differentiation of an inflammatory MΦ phenotype and that RALDH activity in MΦ themselves may contribute to this process. This observation indicates that in IBD one potential role of RALDH activity in human intestinal CD14⁺ MΦ is related to promotion of differentiation toward an ‘inflammatory’ rather than a steady state-associated phenotype.

5:2 Introduction

It was established in Chapter 4 that human intestinal CD14⁺ MΦ possess RALDH activity which is significantly enhanced in IBD patients compared with healthy controls. However, the origin of this activity and how it influences cellular function of CD14⁺ MΦ within the intestinal mucosa remain unclear. Human intestinal CD14⁺ MΦ are implicated

in the pathogenesis of IBD based on a pro-inflammatory profile. They produce TNFα and IL-6 following microbial stimulation (Kamada et al., 2008), both of which are present at increased concentration in CD and UC (reviewed by Strober and Fuss, 2011). CD14⁺ MΦ are sensitive to stimulation through IgG-antigen complexes due to surface expression of CD64 (FcγR1). This induces TNFα and IL-1β production and is important as CD and UC are associated with an increased proportion of LP IgG⁺ plasma cells (Uo et al., 2012). Disease-specific features of CD14⁺ MΦ have also been identified. In CD, these cells produce significantly greater quantities of IL-23 as compared with their UC or healthy control counterparts. This promotes IFNγ production by intestinal LP T and NK cells (Kamada et al., 2008, Takayama et al., 2010). A significantly increased proportion of intestinal MΦ display the pro-inflammatory CD14⁺ phenotype in IBD patients (Grimm et al., 1995a, Kamada et al., 2008, Tamoutounour et al., 2012, Bain et al., 2013). In contrast, the majority of resident intestinal MΦ in healthy control patients are CD14⁻ and do not produce pro-inflammatory cytokines in response to microbial stimulation. They are however able to efficiently phagocytose and kill intracellular bacteria (Smythies et al., 2005). Limited responsiveness to microbial stimulation is the result of a combination of low expression of surface receptors e.g. CD14, as well as suppression of NF-κB signalling (Smythies et al., 2010).

A parallel of the human intestinal CD14⁺ and CD14⁻ MΦ system has been reported in mice; however the subsets are not distinguished by CD14 expression. In the steady state, CX₃CR1^{hi} MΦ display an anti-inflammatory profile and constitutively produce IL-10 which is enhanced following microbial stimulation. In models of intestinal inflammation however, there is an accumulation of a heterogeneous population of CX₃CR1^{int} cells described as inflammatory MΦ (Bain et al., 2013, Tamoutounour et al., 2012) and DCs (Rivollier et al., 2012, Zigmond et al., 2012). They display increased

production of IL-6, IL-1 β and iNOS (Weber et al., 2011b, Zigmond et al., 2012). Both the CX₃CR1^{hi} and CX₃CR1^{int} populations represent alternative differentiation fates of Ly6C^{hi} monocytes (Bain et al., 2013, Tamoutounour et al., 2012, Rivollier et al., 2012, Zigmond et al., 2012).

A similar system is likely to operate in humans as CD14⁺ blood monocytes are linked with both intestinal CD14⁺ and CD14⁻ MΦ phenotypes. Culturing CD14⁺ monocytes *in vitro* with intestinal stromal cell conditioned medium induces a phenotype closely resembling intestinal CD14⁻ MΦ (Smythies et al., 2005). This phenotype includes significantly reduced responsiveness to microbial stimulation alongside maintained phagocytic and bactericidal activity (Smythies et al., 2005). Furthermore, intestinal CD14⁺ MΦ and CD14⁺ blood monocytes share features beyond CD14 expression including expression of CD64, CD11c (Smythies et al., 2005, Kamada et al., 2008, Bain et al., 2013) and the production of reactive oxygen species (Hausmann et al., 2001). The uptake of labelled monocytes into inflamed intestinal mucosa of IBD patients from circulation has also been demonstrated (Grimm et al., 1995b).

It has been proposed that monocytes enter the human intestinal mucosa in the steady by chemotaxis towards IL-8 and TGF- β , produced by stromal cells within the LP (Smythies et al., 2006). In CD and UC there is a significant increase in the expression of a range of chemokines including IL-8 and CCL2 (monocyte chemoattractant protein [MCP]-1) within the intestinal mucosa which induces high levels of monocyte recruitment (Reinecker et al., 1995, Grimm et al., 1996, Banks et al., 2003). Furthermore, CCL2 has also been implicated in promoting a pro-inflammatory CD14⁺ MΦ phenotype in IBD (Spoettl et al., 2006). The elevated proportion of CD14⁺ MΦ in IBD therefore likely reflects accumulation of CD14⁺ monocytes which then fail to undergo differentiation into the steady state resident CD14⁻ MΦ phenotype.

The enhanced RALDH activity of CD14⁺ MΦ in IBD may therefore be related to the pro-inflammatory profile of these cells. RA significantly influences the differentiation of myeloid cells (Breitman et al., 1980). High dose RA is prescribed in the treatment of acute promyelocytic leukaemia (APL) where it induces the differentiation of myeloid progenitor cells (Degos and Wang, 2001). It is therefore hypothesised that RA production by CD14⁺ MΦ through RALDH activity plays a role in determining the differentiation fate of CD14⁺ monocytes, favouring development of the pro-inflammatory CD14⁺ MΦ phenotype.

Murine SI-LP CD103⁺ DCs possess enhanced RALDH activity which is absent in circulating preDCs (Jaensson-Gyllenback et al., 2011). By analogy, it is therefore hypothesised that the enhanced RALDH activity of human intestinal CD14⁺ MΦ develops following entry of these cells into the intestinal mucosa and is not apparent in circulating CD14⁺ monocytes. This question is addressed in the first part of this chapter by analysis of RALDH activity in CD14⁺ monocytes of healthy control and IBD patients. This then allows a direct comparison between RALDH activity of CD14⁺ monocyte and MΦ in these groups.

5:3 Aims

1. Determine whether RALDH activity of CD14⁺ MΦ is acquired within the intestinal mucosa and whether it is locally enhanced in IBD patients by studying RALDH activity in CD14⁺ blood monocytes
2. Determine whether RALDH activity and RA generation play a role in the differentiation of inflammatory macrophages

5:4 Results

5:4.1 Enhanced RALDH activity of CD14⁺ MΦ in IBD is acquired following entry of monocytes into the intestinal mucosa

It was observed in Chapter 4 that the Aldefluor/RALDH activity of CD14⁺ MΦ is enhanced in IBD compared with healthy control patients. However, the origins of this enhanced activity were unknown. Intestinal CD14⁺ MΦ derive from circulating CD14⁺ blood monocytes (Grimm et al., 1995b). Enhanced RALDH activity of intestinal CD14⁺ MΦ in IBD may therefore develop within the intestinal mucosa in response to local mediators, or alternatively may already be present within CD14⁺ monocytes in circulation. In order to distinguish between these possibilities, RALDH activity of CD14⁺ blood monocytes in healthy control and IBD patients was analysed.

CD14⁺ monocytes were identified in PBMCs by flow cytometry on the basis of forward and side-scatter and CD14 expression (Figure 5.1A). Following the Aldefluor assay, approximately 25 % of CD14⁺ monocytes from healthy controls displayed Aldefluor activity that was inhibited in the presence of DEAB (Figure 5.1A). Furthermore, real-time PCR analysis of immuno-magnetically sorted CD14⁺ monocytes identified prominent expression of *ALDH1A1* in these cells (Figure 5.1B). In contrast, *ALDH1A2* was expressed at approximately 1000 fold reduced levels as compared with *ALDH1A1*, whilst *ALDH1A3* was routinely below the limit of detection (Figure 5.1B). Therefore CD14⁺ monocytes from healthy controls, in common with the intestinal CD14⁺ MΦ, display Aldefluor/RALDH activity which associates with *ALDH1A1* expression.

CD14⁺ monocytes from IBD patients also displayed Aldefluor/RALDH activity, with a similar profile of *ALDH1A* expression (Figure 5.1B). However, a significantly reduced proportion of CD14⁺ monocytes from IBD patients were Aldefluor⁺ as compared with

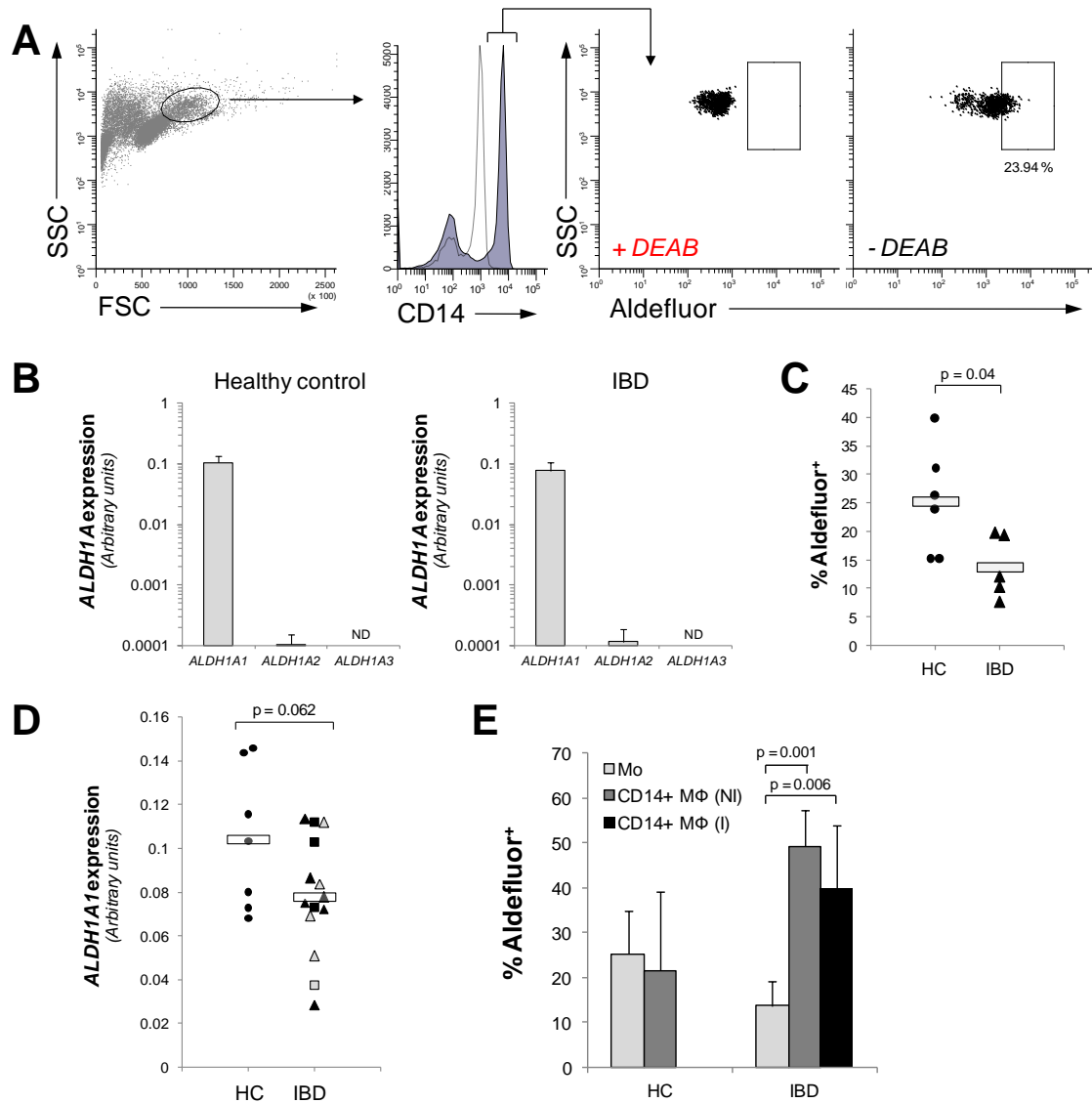


Figure 5.1: Aldefluor/RALDH activity is present in human CD14⁺ blood monocytes and is upregulated following entry into the intestinal mucosa in IBD. **A:** Aldefluor assay was performed on PBMCs and activity was determined in CD14⁺ monocytes by gating on cells in the monocyte region of the FSC vs. SSC plot, then on CD14⁺ cells (filled histogram) as defined in comparison with matched mouse IgG2a isotype control (dotted histogram). Aldefluor⁺ cells were defined in relation to a DEAB-inhibited control reaction as previously, with a fixed separation introduced in the gating to ensure only significantly Aldefluor⁺ cells were analysed. **B:** CD14⁺ monocytes were immuno-magnetically purified from PBMCs from healthy control (n=7) and IBD patients (n=14) with CD14 microbeads; *ALDH1A* expression was determined by real-time PCR. Expression normalized to *GAPDH*; bars indicate mean values + SD, ND is not detected. *Continued on next page...*

...Continued from previous page. **C:** Summary Aldefluor activity of CD14⁺ blood monocytes for healthy controls (n=6) and IBD patients (n=5); bars indicate mean values, p-value from a t-test. **D:** Comparison of *ALDH1A1* expression of purified CD14⁺ monocytes between healthy control (n=7) and IBD patients (n=14). Expression normalized to *GAPDH*; bars indicate mean values, p-value from a t-test, ● = Healthy ▲ = CD, ■ = UC; black and grey shapes for IBD samples indicate active and quiescent disease, respectively. **E:** Comparison of Aldefluor activity of CD14⁺ monocytes as in Figure 5.1C and CD14⁺ MΦ as in Figure 4.13; NI and I are non-inflamed and inflamed, respectively; bars indicate mean values + SD; p-values derive from One Way ANOVA with pair-wise comparisons by Tukey test.

their healthy control counterparts (p=0.04; Figure 5.1C). This was associated with a trend towards reduced *ALDH1A1* expression in CD14⁺ monocytes in IBD patients, although this difference did not reach statistical significance (p=0.062; Figure 5.1D). When compared directly, there was a clear and statistically significant upregulation of Aldefluor/RALDH activity within CD14⁺ MΦ from IBD patients compared with their monocyte precursors (Figure 5.1E). In contrast, the level of RALDH activity did not differ between monocytes and CD14⁺ MΦ from healthy controls. This strongly indicates that CD14⁺ monocytes enter the intestinal mucosa in both healthy control and IBD patients with basal RALDH activity. Whilst in healthy controls this level of activity is maintained, Aldefluor/RALDH activity of CD14⁺ monocytes becomes significantly enhanced following entry into the intestinal mucosa in IBD.

Real-time PCR identified expression of the retinol dehydrogenase enzymes *RDH10* and *DHRS9* in CD14⁺ blood monocytes, with no statistically significant difference in expression between these cells in healthy controls and IBD patients (Figure 5.2). An additional retinol dehydrogenase, *RDH16*, was also expressed by CD14⁺ monocytes at levels approximately 100 fold lower than either *RDH10* or *DHRS9*. Furthermore, *RDH16* was expressed at significantly lower levels in CD14⁺ monocytes from IBD patients compared with their healthy control counterparts (p=0.028; Figure 5.2). Overall

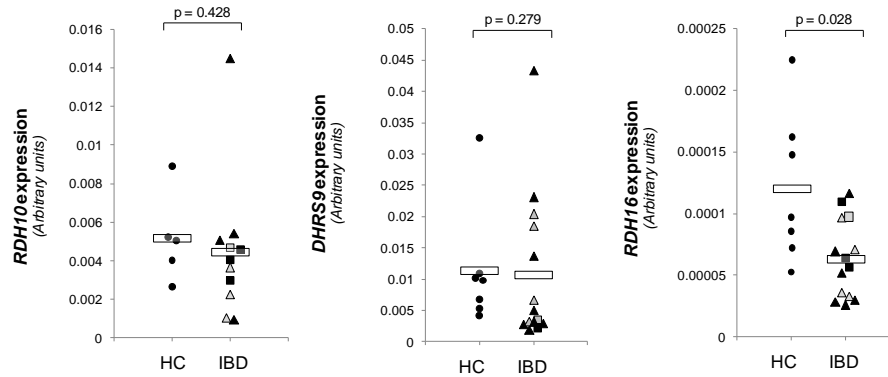


Figure 5.2: CD14⁺ monocytes from healthy control and IBD patients express multiple retinol dehydrogenase enzymes. Expression of *RDH10*, *DHRS9* and *RDH16* was determined in immuno-magnetically purified CD14⁺ monocytes by real-time PCR from healthy controls (*RDH10*: n=5; *DHRS9*/*RDH16*: n=7) and IBD patients (*RDH11*: n=5; *DHRS9*/*RDH16*: n=14). Expression normalized to *GAPDH*; bars indicate mean values, P-values from t-tests, ● = Healthy ▲ = CD, ■ = UC; black and grey shapes for IBD samples indicate active and quiescent disease, respectively.

this indicates that in addition to RALDH activity, CD14⁺ monocytes enter the intestinal mucosa with ability to oxidise retinol to retinal in both healthy control and IBD patients.

The oncogene *MN1* suppresses expression of RAR α -regulated genes in haematopoietic cells (Meester-Smoor et al., 2008, Kandilci and Grosveld, 2009). It was therefore speculated that modified expression of *MN1* may have been responsible for the reduced *RDH16* and trend towards reduced *ALDH1A1* expression in CD14⁺ monocytes from IBD patients. Indeed, *MN1* expression was significantly higher in CD14⁺ monocytes from IBD patients compared with those from healthy controls ($p=0.006$; Figure 5.3). Overall, these data suggest the presence of significant differences in the phenotype of CD14⁺ monocytes between healthy control and IBD patients. These differences may influence the upregulation of RALDH activity in CD14⁺ MΦ in the intestinal mucosa of IBD patients.

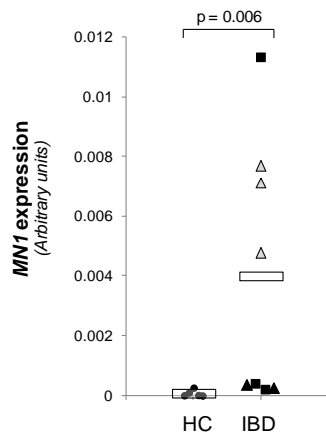


Figure 5.3: CD14⁺ monocytes from IBD patients express enhanced levels of *MN1* compared with CD14⁺ monocytes from healthy controls. Expression of *MN1* was determined in CD14⁺ monocytes immuno-magnetically purified CD14⁺ monocytes by real-time PCR from healthy controls (n=5) and IBD patients (n=8). Expression normalized to *GAPDH*; bars indicate mean values, p-values from t-tests, ● = Healthy ▲ = CD, ■ = UC; black and grey shapes for IBD samples indicate active and quiescent disease, respectively.

5:4.2 RALDH activity in CD14⁺ MΦ may contribute to an inflammatory phenotype

The functional significance of enhanced RALDH activity in CD14⁺ MΦ in IBD remained unclear. Based on an association between RA and myeloid cell differentiation (Breitman et al., 1980), it was hypothesised that the RALDH activity of CD14⁺ MΦ provides RA required to promote differentiation into the CD14⁺ as opposed to the CD14⁺ MΦ phenotype. An *in vitro* system was established to test this hypothesis.

It was observed in Chapter 3 that human CD14⁺ blood monocytes cultured in the presence of GM-CSF for 7 days developed high levels of Aldefluor/RALDH activity. Furthermore, GM-CSF induces the differentiation of human monocytes into pro-inflammatory MΦ which produce cytokines including TNFα, IL-23 and IL-6 in response to stimulation with LPS (Verreck et al., 2004). Based on this pro-inflammatory cytokine profile and the observed RALDH activity, the *in vitro* differentiation of monocytes with GM-CSF was therefore used as a model to study the role of RALDH activity in human intestinal CD14⁺ MΦ.

It was hypothesised that the RA produced by RALDH activity during MΦ differentiation would be required for the development of a pro-inflammatory phenotype. Consistent with the results in Chapter 3, CD14⁺ blood monocytes cultured in GM-CSF for 7 days developed high levels of Aldefluor/RALDH activity (Figure 5.4A). Aldefluor/RALDH activity in these *in vitro*-derived MΦ was significantly greater than that observed in freshly isolated CD14⁺ monocytes from healthy control individuals ($p=0.007$; Figure 5.4C). Furthermore, Aldefluor/RALDH activity was significantly inhibited by inclusion of the RARα antagonist Ro41-5253 throughout monocyte differentiation (Figures 5.4A and B). The *ALDH1A* expression profile of the DMSO-treated MΦ was similar to CD14⁺ monocytes, with predominant expression of *ALDH1A1* (Figure 5.4D). Finally, MΦ grown in the presence of Ro41-5253 displayed a significant reduction in the level of *ALDH1A1* expression ($p<0.001$; Figure 5.4E). Overall, this confirms that CD14⁺ monocytes cultured with GM-CSF develop significant levels of Aldefluor/RALDH activity which closely associates with *ALDH1A1* expression and is suppressed in the absence of RARα signalling. This is consistent with a model where RALDH activity generates RA, which then acts in a positive feedback loop to enhance RALDH activity further.

Following 6 day culture of CD14⁺ monocytes with GM-CSF, the majority of cells remained CD14⁺. In contrast, a significantly reduced proportion were CD14⁺ when cultured with GM-CSF in the presence of Ro41-5253 ($p<0.001$; Figures 5.5A and B). Furthermore, Ro41-5253-treated MΦ which remained CD14⁺ displayed significantly increased Aldefluor/RALDH activity compared with their CD14⁺ counterparts ($p=0.018$; Figure 5.5C). This observation demonstrates an association between loss of RALDH activity and CD14 expression at the single cell level and indicates that RA plays an important role in maintaining CD14 expression in human MΦ. RALDH activity may therefore provide an important source of RA for this process. In addition to modified

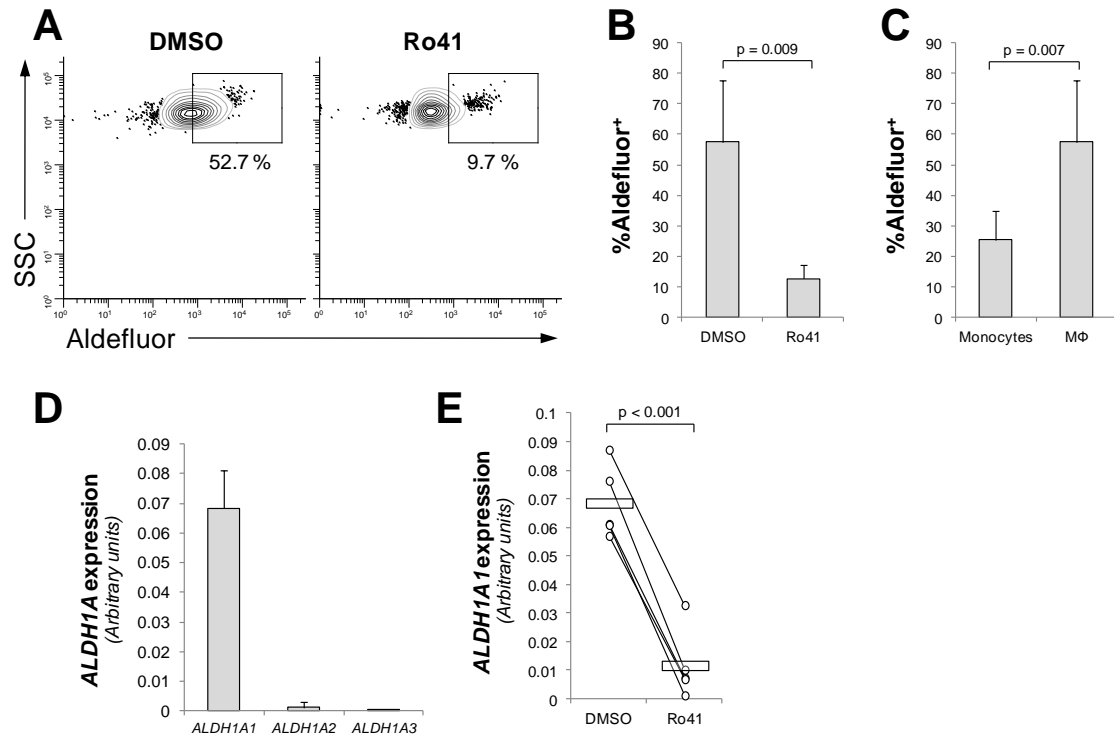


Figure 5.4: GM-CSF induces Aldefluor/RALDH activity in human CD14⁺ monocytes which is suppressed in the absence of RAR α signalling. **A:** CD14⁺ monocytes were cultured in GM-CSF (100 ng/ml) for 6 days in the presence of Ro41-5253 (1 μ M) or concentration-matched DMSO (vehicle control) and analysed by Aldefluor assay. Gating defined relative to DEAB-inhibited control (not shown); per cent Aldefluor^{hi} cells indicated in each case. **B:** Summary Aldefluor activity of MΦ treated with Ro41-5253 or DMSO (n=5). **C:** Comparison of Aldefluor activity of MΦ (treated with DMSO; as in Figure 5.4B) with healthy control CD14⁺ monocytes (as in Figure 5.1C). **D:** CD14⁺ monocytes from healthy controls were immuno-magnetically purified using CD14 microbeads and *ALDH1A* expression profile determined by real-time PCR. **E:** Summary *ALDH1A1* expression data of MΦ treated with Ro41-5253 and DMSO (vehicle control). Expression normalized to *RPL30*, bars indicate mean values + SD; p-values derive from paired t-tests, except Figure 5.4C (t-test).

expression of CD14, the overall level of HLA-DR expression was also reduced in MΦ treated with Ro41-5253 ($p=0.007$; Figures 5.5A and B).

Having established a clear association between the loss of RALDH activity and surface CD14 expression, the next step was to establish whether this was also related to a reduced pro-inflammatory phenotype. To address this, Ro41-5253 and DMSO-treated

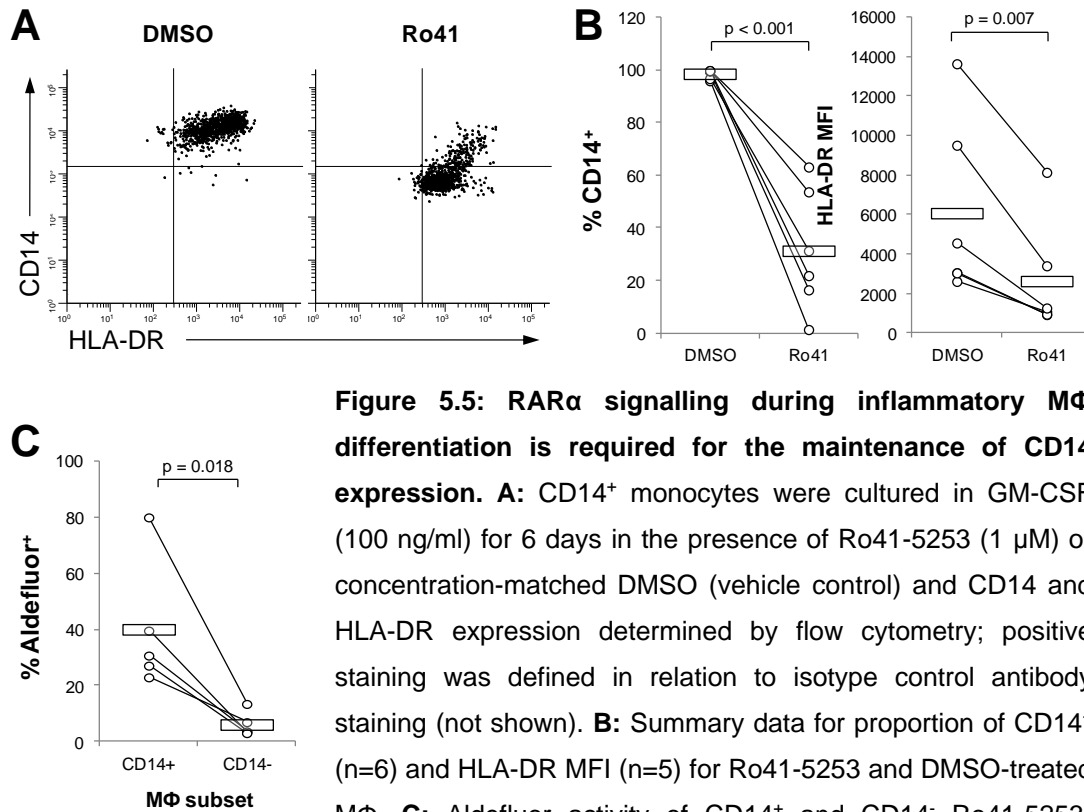


Figure 5.5: RAR α signalling during inflammatory MΦ differentiation is required for the maintenance of CD14 expression. **A:** CD14⁺ monocytes were cultured in GM-CSF (100 ng/ml) for 6 days in the presence of Ro41-5253 (1 μ M) or concentration-matched DMSO (vehicle control) and CD14 and HLA-DR expression determined by flow cytometry; positive staining was defined in relation to isotype control antibody staining (not shown). **B:** Summary data for proportion of CD14⁺ (n=6) and HLA-DR MFI (n=5) for Ro41-5253 and DMSO-treated MΦ. **C:** Aldefluor activity of CD14⁺ and CD14⁻ Ro41-5253-treated MΦ (n=5). Bars indicate mean values; p-values derive from paired t-tests.

MΦ were stimulated with LPS for 4.5 hours and the accumulation of intracellular TNF α was determined by intracellular flow cytometry. Neither group produced significant TNF α in absence of stimulation. However, following LPS stimulation the majority of DMSO-treated MΦ were TNF α ⁺ (90.6 \pm 5.9 %; Figure 5.6A). In contrast, a significantly reduced proportion of Ro41-5253-treated MΦ were TNF α ⁺ (29.6 \pm 18.7 %; p=0.003; Figures 5.6A and B). Whilst the proportion of MΦ producing IL-10 following LPS stimulation was considerably lower than that of TNF α , there was also a trend towards reduced IL-10 production by Ro41-5253-treated MΦ (p=0.079; Figure 5.6C).

The reduced production of TNF α by Ro41-5253-treated MΦ could have been the result of diminished cell viability. To address this, the proportion of cells that were both negative for the dead cell marker 7-aminoactinomycin (7-AAD) and large enough (by

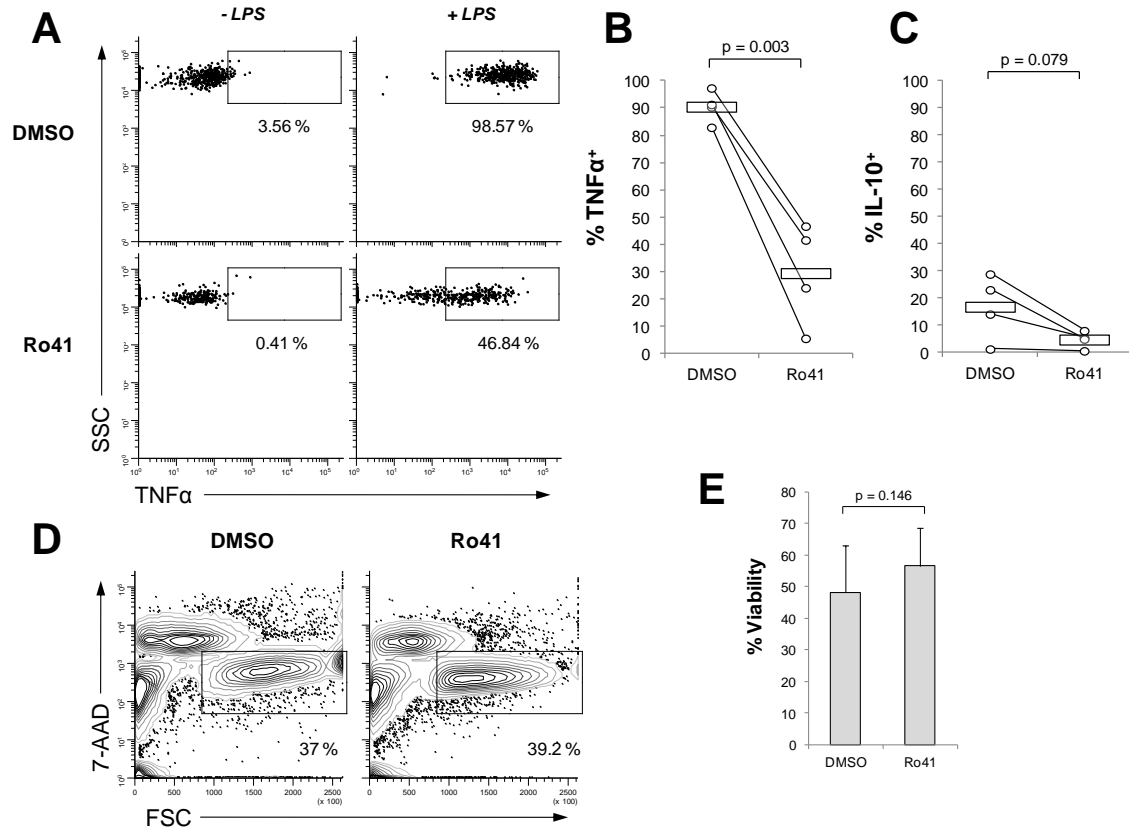


Figure 5.6: RARα signalling during human inflammatory MΦ differentiation is required for the ability to produce TNFα in response to LPS stimulation. **A:** CD14⁺ monocytes were cultured in GM-CSF (100 ng/ml) for 6 days in the presence of Ro41-5253 (1 μM) or concentration-matched DMSO (vehicle control) and then cultured in the presence or absence of LPS (1 μg/ml) for 4.5 hours, and TNFα production determined by intracellular flow cytometry; proportion of TNFα⁺ cells indicated in each case. **B:** Summary data for intracellular TNFα (n=4) as in Figure 5.5A. **C:** Summary data for intracellular IL-10 (n=4) determined as for TNFα in Figure 5.5A. **D:** Viability of Ro41-5253 and DMSO-treated MΦ as defined by lack of 7-AAD staining and sufficient forward scatter (FSC) to exclude cellular debris; proportion of viable cells noted. **E:** Summary viability data for Ro41-5253 and DMSO-treated MΦ (n=5); bars indicate mean values + SD; P-values derive from paired t-tests.

forward scatter) to exclude cellular debris was compared between the MΦ groups (Figure 5.6D). No significant difference could be identified the level of cell viability between DMSO and Ro41-5253-treated MΦ (Figures 5.6D and E). Overall, this

indicates that RA signalling through RAR α is required during the differentiation of human inflammatory MΦ *in vitro* for the development of RALDH activity, maintenance of CD14 expression and ability to produce TNF α in response to LPS. RALDH activity may therefore contribute to this process through the provision of RA. This is consistent with a role for RALDH activity in human intestinal CD14⁺ MΦ in the development of a pro-inflammatory phenotype in IBD.

5:5 Discussion

The first aim of this chapter was to establish the origin of enhanced RALDH activity in CD14⁺ MΦ in IBD. As intestinal CD14⁺ MΦ derive from circulating CD14⁺ monocytes (Grimm et al., 1995b), the first step was to determine whether enhanced RALDH activity was intrinsic to CD14⁺ monocytes in IBD patients or else followed entry of these cells into the intestinal mucosa. Basal levels of Aldefluor/RALDH activity were observed in CD14⁺ monocytes from both healthy control and IBD patients, indicating that monocytes enter the distal human intestinal mucosa with RALDH activity which is then selectively upregulated in IBD patients. Furthermore, expression of *RDH10* and *DHRS9* by CD14⁺ monocytes indicates that these cells enter the intestinal mucosa with the complete enzymatic machinery to produce RA from retinol.

The *ALDH1A* expression profile of CD14⁺ monocytes from both healthy control and IBD patients was dominated by *ALDH1A1*, with approximately 1000 fold lower expression of *ALDH1A2* and *ALDH1A3* below the limit of detection. As seen in Chapter 4, *ALDH1A1* was also the predominant *ALDH1A* isoform in intestinal CD14⁺ MΦ but was not detected in intestinal DCs. This is consistent with a direct precursor-product relationship between human CD14⁺ monocytes and intestinal CD14⁺ MΦ but not between CD14⁺ monocytes and intestinal DCs. Furthermore, it has been demonstrated that murine SI-LP CX₃CR1^{hi} MΦ in the steady state and CX₃CR1^{int} inflammatory MΦ in models of IBD derive from Ly6C^{hi} monocytes (Bain et al., 2013, Tamoutounour et al., 2012, Rivollier et al., 2012, Zigmond et al., 2012) whereas CD103⁺ DCs derive from committed DC progenitors (Bogunovic et al., 2009). *ALDH1A1* expression may therefore act as a marker of monocyte-derived cells in the human intestinal mucosa.

CD14⁺ monocytes from IBD patients displayed significantly reduced Aldefluor/RALDH activity compared with their counterparts in healthy controls. The functional significance and the mechanism behind this observation however remain unclear. RALDH1 in rats is inhibited by apo-CRBP-I, which is an indicator of low cellular retinol concentration (Posch et al., 1992). In addition, serum retinol is reduced in IBD patients (Janczewska et al., 1991). Theoretically this could lead to reduced pairing of retinol and CRBP-I in CD14⁺ monocytes and therefore inhibition of RALDH1, reducing overall Aldefluor/RALDH activity. Alternatively, reduced activity could be the result of the observed trend towards reduced *ALDH1A1* expression in CD14⁺ monocytes in IBD patients.

The role of RALDH1 activity in human CD14⁺ monocytes is unclear. As discussed in Chapter 3, the Aldefluor assay was originally developed for the identification and isolation of viable haematopoietic stem cells (HSCs) on the basis of ALDH activity and low side scatter (Storms et al., 1999). As monocytes ultimately derive from HSCs following a differentiation process within the bone marrow (Auffray et al., 2009), the RALDH1 activity observed in monocytes may be preserved from an earlier stage of differentiation. There are indications in mice that *Aldh1a1* expression and ALDH activity in HSCs are linked with a promotion of myeloid versus lymphoid differentiation (Rice et al., 2008, Storms et al., 2005). This suggests that the RALDH activity of CD14⁺ monocytes is the result of a process favouring monocyte differentiation. However, it has been also observed that *Aldh1a1*^{-/-} mice display no defects in the differentiation of myeloid or lymphoid cells (Levi et al., 2009), which may indicate redundancy in the provision of RA by RALDH enzymes during myeloid cell differentiation.

Reduced expression of *ALDH1A1* in human CD14⁺ MΦ may therefore indicate important differences in the differentiation of these cells in IBD patients. Consistent with

this hypothesis, CD14⁺ monocytes expressed significantly greater levels of *MN1* than their healthy control counterparts. Differentiation of human CD34⁺ bone marrow HSCs to either CD14⁺ monocytes or CD15⁺ granulocytes associates with a significant downregulation of *MN1* (Kandilci and Grosveld, 2009). Furthermore, *MN1* overexpression is observed in some forms of acute myeloid leukaemia (AML) where it promotes proliferation as opposed to differentiation of myeloid progenitors (Grosveld, 2007). Therefore, elevated *MN1* expression by CD14⁺ monocytes in IBD may indicate reduced differentiation of these cells. *MN1* can both promote and repress transcription of RAR-regulated genes including *DHRS9* when overexpressed in a pro-monocytic cell line (Meester-Smoor et al., 2008). Based on the requirement for RAR α signalling in *ALDH1A1* expression as observed in both this Chapter and Chapter 3, elevated *MN1* expression could be responsible for the trend towards suppressed *ALDH1A1* expression in CD14⁺ monocytes in IBD.

Overexpression of *Mn1* in murine bone marrow cells leads to an accumulation of committed myeloid progenitors (CMPs) with suppressed expression of both *Csf1r* (CD115) and *Csf2r* (CD116), the receptors for M-CSF and GM-CSF, respectively (Kandilci et al., 2013). Circulating monocytes and granulocytes in IBD patients have significantly reduced expression of CD116 but not CD115 (Goldstein et al., 2011). Whilst the mechanism behind this differential expression is unknown, it may relate to the modified *MN1* expression observed here. Finally, monocyte-derived MΦ from CD patients display reduced responses to TLR stimulation which may relate to a reduced level of differentiation (Smith et al., 2009, Sewell et al., 2012).

As observed in Chapter 4, intestinal CD14⁺ MΦ in IBD patients express significantly greater levels of *ALDH1A1* as compared with their healthy control counterparts. This likely contributes to the increased overall Aldefluor/RALDH activity of these cells in IBD.

However, comparison of the *ALDH1A* profiles of CD14⁺ monocytes and intestinal CD14⁺ MΦ from both healthy controls and IBD patients identifies an increased prominence of *ALDH1A2* and *ALDH1A3* expression within intestinal CD14⁺ MΦ. Each of these enzyme isoforms may therefore contribute to the net Aldefluor/RALDH activity of CD14⁺ MΦ. Furthermore, post-transcriptional regulation of *ALDH1A* expression may also play a significant role in determining the ability of CD14⁺ MΦ to generate RA within the intestinal mucosa.

As demonstrated in both this Chapter and Chapter 3, GM-CSF is a potent inducer of RALDH activity in human CD14⁺ monocytes. This is therefore a candidate for the upregulation of RALDH activity in human intestinal CD14⁺ MΦ in IBD. Consistent with this hypothesis, GM-CSF is present at a significantly increased concentration in the colonic mucosa of both CD and UC patients compared with healthy controls (Ina et al., 1999). As discussed earlier, the GM-CSF receptor (CD116) is expressed at a significantly lower level on both granulocytes and monocytes from IBD patients (Goldstein et al., 2011). This indicates a modified relationship between CD14⁺ monocytes and GM-CSF signalling in IBD patients and healthy controls. Whether reduced expression of CD116 influences the differentiation of CD14⁺ monocytes following entry into the intestinal mucosa is unclear. Moreover, the extent to which CD14⁺ MΦ upregulate CD116 expression within the mucosa and whether this modulates RALDH activity is unknown, and warrants further attention.

An alternative candidate for the upregulation of RALDH activity in CD14⁺ MΦ in IBD is RA itself. In the mouse, exposure of murine CD103⁺ DCs to RA is directly responsible for upregulation of *Aldh1a2* expression and RALDH activity (Villablanca et al., 2011b, Jaensson-Gyllenback et al., 2011). Furthermore, the presence of exogenous RA enhances the RALDH activity of human MoDCs as demonstrated in Chapter 3.

However, the level of RA present within the intestinal mucosa of IBD and healthy control patients was very similar, as demonstrated in Chapter 4. This indicates that whilst RA is likely to be required for the upregulation of RALDH activity in CD14⁺ MΦ in IBD, it is unlikely to be the principal driver of the enhancement of this activity in IBD.

RALDH activity of human MoDCs is enhanced following exposure to various TLR ligands (Villablanca et al., 2011b, Wang et al., 2011b). As demonstrated in Chapter 3, stimulation of human MoDCs with LPS also induced a slight increase in RALDH activity which was due to an increase in the proportion of cells with a mature phenotype. The increased RALDH activity of CD14⁺ MΦ in IBD may therefore be due to exposure to an array of microbial products not present in healthy control patients. This is consistent with the increased intestinal permeability associated both CD and UC (reviewed by Baumgart and Sandborn, 2012, Ordas et al., 2012).

It was hypothesized that the upregulation of RALDH activity by human intestinal CD14⁺ MΦ in IBD patients was associated with the differentiation of CD14⁺ monocytes following entry into the intestinal mucosa. This view was suggested by the established role of RA in cellular differentiation, including the differentiation of myeloid cells (Zhang et al., 2000). An *in vitro* system was developed to address this possibility.

Culture of CD14⁺ monocytes from healthy controls in the presence of GM-CSF generated cells with multiple features shared with intestinal CD14⁺ MΦ in IBD patients. These included enhanced Aldefluor/RALDH activity and maintained expression of CD14, as demonstrated in this Chapter, as well as a uniform ability to produce TNFα in response to LPS stimulation (Verreck et al., 2004). In order to determine the role of RA production in this system, cells were cultured in the presence of the RARα antagonist Ro41-5253 (Apfel et al., 1992) throughout differentiation. This resulted in significantly

reduced Aldefluor/RALDH activity alongside suppressed *ALDH1A1* expression. Furthermore, expression of CD14 and the ability to produce TNFα following LPS stimulation were also significantly reduced. The ability of Ro41-5253 to significantly influence the GM-CSF-mediated differentiation of blood CD14⁺ monocytes is consistent with the constitutively high level expression of RARα and a key heterodimeric partner RXRα in these cells as observed elsewhere (Fritsche et al., 2000, Szatmari et al., 2006).

The generation of pro-inflammatory CD14⁺ and less inflammatory CD14⁻ MΦ in this system mirrors intestinal CD14⁺ and resident CD14⁻ MΦ phenotypes. It also establishes a clear association between RALDH activity and a pro-inflammatory MΦ phenotype. However, a limitation of this system is that Ro41-5253 suppresses the signalling of both RA generated by RALDH during MΦ differentiation as well as RA endogenous to the cell culture media. Further experiments are therefore required to distinguish the specific contribution of RALDH-derived RA to this process. The system could be developed by culturing CD14⁺ monocytes with GM-CSF in the presence of DEAB to inhibit RALDH activity throughout differentiation. Alternatively, *ALDH1A1* expression could be suppressed artificially using siRNA technology.

The *in vitro* MΦ differentiation system identified a clear role for RARα in the maintenance of CD14 expression. The requirement of RARα signalling for the expression of both *ALDH1A1* and CD14 may be related to the transcription factor C/EBPβ, which is also implicated in the expression of these genes (Pan et al., 1999, Elizondo et al., 2009). Furthermore C/EBPβ plays a key role in the differentiation of monocytes into MΦ in mouse and man reviewed by Huber et al., 2012). It is therefore hypothesised that inhibition of RARα signalling during the differentiation of CD14⁺ monocytes leads to a reduction in C/EBPβ activity and subsequently a loss of CD14

expression. The elevated Aldefluor/RALDH activity of CD14⁺ compared with CD14⁻ Ro41-5253-treated MΦ is consistent with a link between RA production and signalling and retention of CD14 expression.

The reduced production of TNFα following stimulation of Ro41-5253-treated MΦ with LPS may simply reflect reduced sensitivity based on the lack of surface CD14 expression, which could be addressed through stimulation of these cells through alternative TLR ligands. Alternatively, this could reflect a more general suppression of inflammatory activity as observed with intestinal CD14⁻ MΦ (Smythies et al., 2005). C/EBPβ promotes NF-κB signalling in mice through reduced transcription of IκBα, an inhibitor of the nuclear translocation of NF-κB (Cappello et al., 2009). Furthermore, *Cebpb*^{-/-} mice are more sensitive to bacterial infections including *Listeria monocytogenes* (Tanaka et al., 1995). C/EBPβ has also been shown to bind to the human TNFα promoter directly (Pope et al., 1994). C/EBPβ may therefore also play a key role in the differentiation of CD14⁺ MΦ within the intestinal mucosa in IBD. It has been demonstrated that C/EBPβ regulates FcγR-mediated inflammatory responses to IgG-antigen immune complexes in murine MΦ (Yan et al., 2012). Human CD14⁺ MΦ are also stimulated in the presence of IgG immune complexes, producing TNFα and IL-1β (Uo et al., 2012).

Overall, this is consistent with a role for C/EBPβ in the differentiation of human inflammatory CD14⁺ MΦ *in vitro*. Modulation of C/EBPβ activity during this process by inhibition of RARα signalling would provide a novel insight into this process. There is currently limited evidence of a direct link between RARα and C/EBPβ expression. One study showed that RA induces C/EBPβ expression and activity in acute promyelocytic leukaemia, but only in the presence of the PML-RARα fusion protein as opposed to conventional RARα (Duprez et al., 2003).

It was recently reported that *Aldh1a2* expression and RALDH2 activity are significantly upregulated in MΦ in the livers of mice infected with the helminth *Schistosoma mansoni* (Broadhurst et al., 2012). Interestingly, this was associated with an alternatively activated MΦ phenotype, typified by expression of arginase1, Ym1 and FIZZ1. Furthermore, in vitamin A-deficient mice there was a significantly reduced T_H2 response in the liver indicated by reduced presence of the cytokines IL-4, IL-5 and IL-13, as well as diminished eosinophil granuloma formation. The upregulation of *Aldh1a2* could be replicated in bone-marrow derived MΦ with IL-4. Collectively, these data indicate that in the mouse, upregulation of *Aldh1a2* in MΦ is associated with an effective T_H2 response in *S. mansoni* infection. Whether the upregulation of this activity is required for the differentiation of these MΦ, i.e. an 'intrinsic' role for RA in this case, is unclear. This does however indicate that RALDH activity is an important component of monocyte differentiation in a range of different contexts.

An important alternative role for RALDH activity in human intestinal CD14⁺ MΦ may be in influencing mucosal T cell responses. It has been demonstrated CD14⁺ MΦ efficiently activate naive CD4⁺ T cells and primarily induce IFN γ production associated with a T_H1 response (Kamada et al., 2009). The lack of CCR7 expression by CD14⁺ observed in Chapter 4 suggests that these cells are unlikely to encounter naive T cells within draining lymphoid tissue. However they are likely to interact with local effector/memory T cell subsets within the intestinal mucosa. The nature of this interaction and how it may be influenced by the RALDH activity of intestinal CD14⁺ MΦ in IBD is an important area for future study. T cells within the intestinal mucosa of mice display a significant degree of plasticity which may allow effector responses to be context-specific (reviewed by Zhou et al., 2009). In this way, Foxp3⁺ T_{REG} may switch phenotype to T_H17 cells in the presence of proinflammatory cytokines including IL-6

(Yang et al., 2008c). RA produced by human intestinal CD14⁺ MΦ may therefore influence such plasticity, perhaps stabilising the FOXP3⁺ T_{REG} phenotype. The elevated proportions of Foxp3⁺ T_{REG} observed in the intestinal mucosa of CD and UC patients compared with healthy controls may be consistent with local provision of RA by CD14⁺ MΦ within these sites (Makita et al., 2004, Maul et al., 2005, Uhlig et al., 2006).

5:6 Conclusion

In conclusion, this chapter has demonstrated that circulating CD14⁺ monocytes in healthy controls and IBD patients have basal levels of RALDH activity which is further enhanced following entry into the distal intestinal mucosa of IBD patients but not healthy controls. It also demonstrates a key role for RA in the differentiation of inflammatory MΦ *in vitro*, including maintenance of expression of CD14 and production of TNFα in response to LPS stimulation. Overall, this is consistent with a role for enhanced RALDH activity in CD14⁺ MΦ in the provision of RA required for the differentiation into this phenotype as opposed to the CD14⁻ MΦ associated with the steady state. Understanding the factors that influence the fate of CD14⁺ monocytes in the intestinal mucosa in IBD may provide new therapeutic options in the treatment of IBD. In particular, treating CD14⁺ monocytes so that they adopt the steady state resident CD14⁻ phenotype within the context of inflammatory mediators in the mucosa of IBD patients may help to clear ongoing microbial presence without the production of inflammatory cytokines and so restore intestinal homeostasis.

Chapter 6: Analysis of the influence of human intestine-derived soluble factors on RALDH expression and activity in DCs

6:1 Chapter summary

This chapter focuses on determining the influence that soluble factors within the intestinal mucosa of healthy control and IBD patients exert on the RALDH activity of human MoDCs. This is particularly important in light of observations in Chapter 4 that the RALDH activity of CD103⁺ DCs and CD14⁺ M Φ is increased in IBD. Contrary to expectation, intestinal biopsy-derived conditioned medium (CM) is suppressive of RALDH activity in MoDCs, at least partially due to the presence of PGE₂. Nonetheless, MoDCs grown in the presence of inflamed IBD intestinal biopsy-derived CM possess an enhanced ability to induce $\alpha_4\beta_7$ on CD4⁺ T cells as compared with non-inflamed IBD biopsy-derived CM treated MoDCs. This effect is independent of RALDH activity and has important implications in how the ability of APCs within the intestinal mucosa of IBD patients to propagate RA signalling in T cells and in particular how the induction of a 'gut-homing' $\alpha_4\beta_7$ phenotype is influenced in the context of inflammation.

6:2 Introduction

In Chapter 4 it was observed that human intestinal CD103⁺ DCs from IBD patients possess enhanced levels of activity in the Aldefluor assay compared with healthy controls. Although this difference did not reach statistical significance, it indicated that contrary to expectation such cells possess enhanced rather than suppressed RALDH activity in IBD. This was supported by the expression of *ALDH1A2* as observed by real-

time PCR of CD103⁺ DCs purified from a CD patient. Due to the low numbers of DCs and the paucity of suitable tissue this approach could not be taken further within the time-frame of this project. In order to circumvent these limitations, an *in vitro* system was established and is the focus of this chapter.

It was hypothesized that factors responsible for modifying RALDH activity of CD103⁺ DCs in IBD would be present as soluble mediators within the tissue. One method to collect such soluble factors is to culture intestinal biopsies in complete medium to generate a biopsy-conditioned medium (CM). The duration of this culture (6 hours) represents a balance between sufficient time to collect significant quantities of cytokines and other soluble mediators in the medium against excessive levels of cell death within the biopsy leading to an increasingly artificial cytokine profile. Differentiation of DCs in the presence of this intestinal biopsy CM therefore allows the influence of soluble factors in healthy control or IBD intestinal mucosa on RALDH activity of DCs to be studied.

MoDCs were used as a substitute for CD103⁺ DCs in this system as they could be reliably generated under control conditions and in sufficient quantities for analysis of function. Moreover, as discussed in Chapter 3, it was already established that they possessed Aldefluor activity that could be directly linked with RALDH activity. This would mean that any modification of RALDH activity by CM could be clearly identified.

The overall influence of intestinal biopsy-conditioned medium (CM) on MoDC RALDH activity may be the result of a balance between factors that enhance or decrease RALDH expression or activity. Well established factors that induce or enhance RALDH activity include RA, acting in a positive feedback loop as observed in Chapter 3 and elsewhere (Villablanca et al., 2011b, Molenaar et al., 2011). GM-CSF has been shown

to induce RALDH activity in murine DCs (Ganguly et al., 2011b) and in combination with IL-4 (Yokota et al., 2009), as observed in Chapters 3 and 5 of this thesis. Stimulation of murine splenic DCs with the TLR2 agonists Pam3CSK4 and zymosan also induce *Aldh1a2* expression and RALDH activity (Wang et al., 2011c, Manicassamy et al., 2009). Moreover, Pam3CSK4 also stimulates RALDH activity in human MoDCs (Villablanca et al., 2011b).

Prostaglandin E₂ (PGE₂) was recently identified as a negative RALDH factor in both murine and human MoDCs (Stock et al., 2011). PGE₂ was shown to stimulate production of cyclic adenosine monophosphate (cAMP), which in turn activates inducible cAMP early repressor (ICER). This then directly inhibits expression of *Aldh1a2* and so suppresses RALDH activity. PGE₂ is known to play a wide range of roles within the intestine, including influencing motility and secretions (Stenson, 2007). Prostaglandins are produced by cyclooxygenase (COX) enzymes found within the intestinal epithelium from arachidonic acid. COX-1 is constitutively expressed within the human intestinal epithelium in the crypt regions, whilst COX-2 expression is induced in the epithelium in both colon cancer and IBD. This upregulation may be mediated by IL-1 β , TNF α and LPS (Stenson, 2007).

The ability of intestinal DCs to imprint an $\alpha_4\beta_7^+$ CCR9⁺ 'gut-homing' phenotype on the CD4⁺ T cells they activate is dependent upon RA (Iwata et al., 2004). Expression of $\alpha_4\beta_7$ allows lymphocytes to interact with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) which is expressed constitutively on vascular endothelial cells in the intestine. Furthermore, CCR9 expression allows lymphocytes to respond to CCL25, which in the gastro-intestinal tract is expressed primarily by small intestinal epithelial cells in the mouse and becomes trapped on vascular endothelial cells (Sigmundsdottir and Butcher, 2008, Agace, 2010). RA has also been shown to suppress induction of E-

selectin ligands on T cells in mice, which are required for migration into non-intestinal sites including the skin during inflammation (Iwata et al., 2004). The human equivalent is cutaneous lymphocyte antigen (CLA), a P-selectin glycoprotein-1 core which is post-translationally modified by $\alpha(1,3)$ -fucosyltransferase VII (Fuhlbrigge et al., 1997).

The close association between provision of RA during naive CD4⁺ T cell activation and the induction of an $\alpha_4\beta_7^+$ CCR9⁺ phenotype therefore provides a functional assay in which the impact of modified RALDH activity in intestinal biopsy CM-treated MoDCs may be assessed. The overall hypothesis for this system is that treatment of human MoDCs with CM derived from healthy control and IBD patients will recapitulate the slightly enhanced levels of RALDH activity in CD103⁺ DCs and the significantly enhanced RALDH activity of CD14⁺ M Φ in IBD. This would lead to an increased generation of RA, which in turn will lead to enhanced induction of $\alpha_4\beta_7$ and CCR9 on naive CD4⁺ T cells with suppression of CLA expression during antigen presentation.

6:3 Aims

1. Determine impact of CM derived from intestinal biopsies of healthy control and IBD patients on RALDH activity in MoDCs
2. Identify factors within CM responsible for modified RALDH activity in MoDCs
3. Assess impact of CM treatment on RALDH-dependent induction of $\alpha_4\beta_7$ and CCR9 on naive CD4⁺ T cells by MoDCs

6:4 Results

6:4.1 Intestinal biopsy conditioned medium suppresses RALDH activity of human MoDCs

As discussed in Chapter 3, human blood CD14⁺ monocytes cultured in GM-CSF and IL-4 differentiate into MoDCs with RALDH activity detectable by Aldefluor assay and consistent with their expression of *ALDH1A2*. When cultured with CFSE-labelled naive CD4⁺ T cells, MoDCs were capable of activating and inducing proliferation of T cells as indicated by alterations in scatter profile (Figure 6.1A) as well as CFSE dilution (Figure 6.1B). This observation confirms that the MoDCs grown *in vitro* possessed naive T cell stimulatory capacity, a key feature of DCs.

In order to understand the influence of the intestinal mucosal environment on RALDH activity of MoDCs, these cells were differentiated in the presence of intestinal biopsy-conditioned medium (CM) at 20 % v/v. To generate CM, intestinal biopsies were

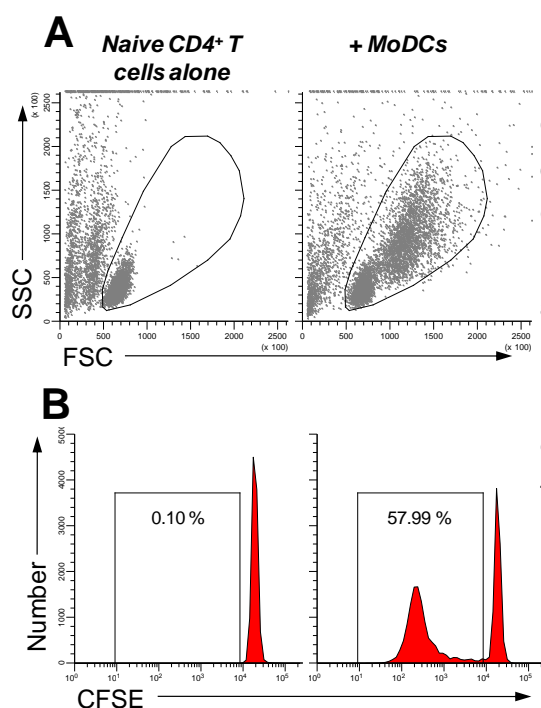


Figure 6.1: Monocyte-derived DCs activate naïve CD4 T cells. CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days then co-cultured with CFSE-labelled naïve CD4⁺ T cells for 5 days at a ratio of 1:10. **A:** Proliferation of CD4⁺ T cells is evident in alteration in forward (FSC) and side-scatter (SSC) profiles. **B:** Proliferating cells display 'diluted' CFSE signal as each subsequent generation of cells inherits half CFSE present in the parent cell. Percent 'CFSE^{lo}' cells shown; representative example (n=20).

collected from patients undergoing endoscopy and cultured in complete medium for 6 hours at 37 °C. CM-treated MoDCs displayed consistently reduced Aldefluor activity compared with non-conditioned MoDCs (Figures 6.2A and B). This reduction was seen with CM generated from both healthy control and from both non-inflamed and inflamed mucosa from IBD patients (Figure 6.2A). Due to considerable variation in the level of Aldefluor activity within non-conditioned MoDCs, a relative Aldefluor activity was determined by dividing the proportion of Aldefluor^{hi} CM-treated MoDCs by the proportion of Aldefluor^{hi} untreated ('non-conditioned') MoDCs in parallel cultures. Comparison of relative Aldefluor activities indicated that CM derived from healthy control and IBD patients significantly suppressed Aldefluor activity of human MoDCs, but there were no statistically significant differences between the different CM groups (Figure 6.2C). Furthermore, similar suppression of RALDH activity was observed with CM derived from both CD and UC patients (Figure 6.2C).

Consistent with the Aldefluor data, CM-treated MoDCs displayed reduced expression of *ALDH1A2*, with no statistically significant separation between different CM groups (Figure 6.3). This is consistent with CM suppressing RALDH2 activity through down-regulation of *ALDH1A2* expression. *ALDH1A1* expression displayed more variation, with expression suppressed in three of four CM-treated MoDCs tested, but enhanced in one sample (Figure 6.3). This is consistent with data in Chapter 3 that indicates *ALDH1A2* expression correlates more strongly than *ALDH1A1* expression with Aldefluor activity of human MoDCs.

Suppression of *ALDH1A2* expression and Aldefluor activity by CM strongly suggested that RALDH2 activity was reduced, and that therefore output of RA by the cells would also be diminished. To confirm this, non-conditioned and CM-treated MoDCs were

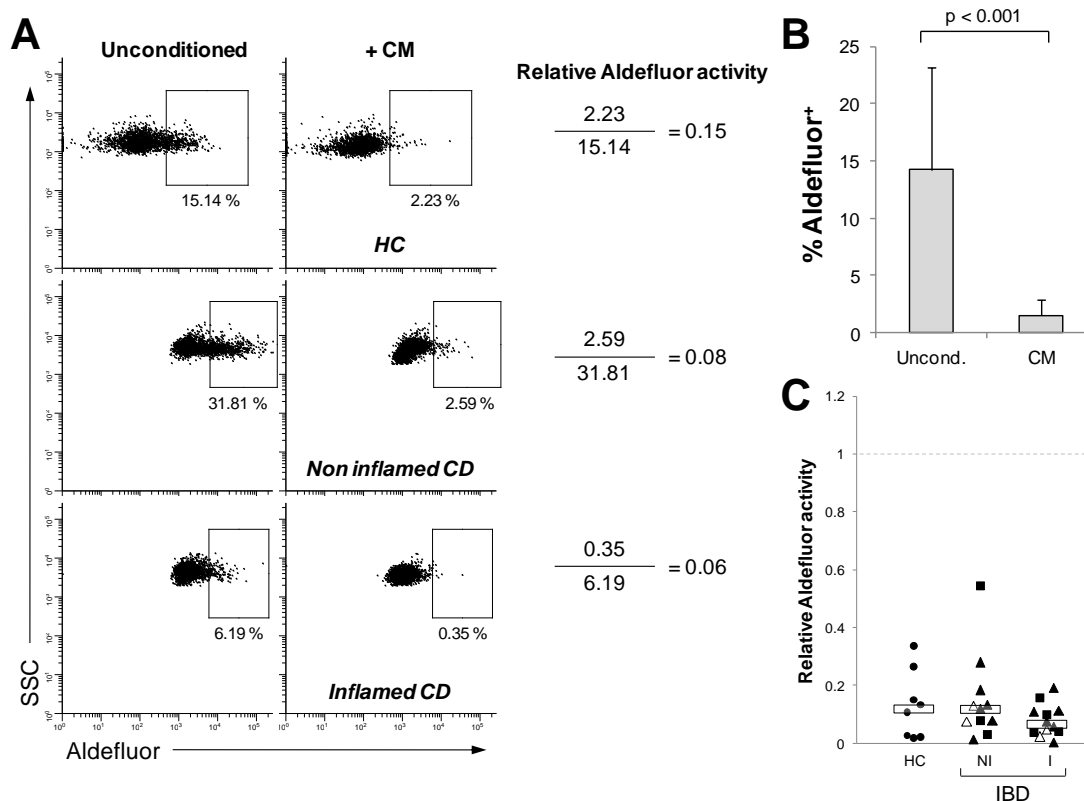


Figure 6.2: Human intestinal biopsy-derived CM suppresses Aldefluor activity of MoDCs. **A:** CD14⁺ monocytes were cultured with GM-CSF and IL-4 (100 ng/ml) in the presence ('+CM') or absence ('Unconditioned') of intestinal biopsy derived-CM, with Aldefluor analysis performed on day 7. Gate defines Aldefluor^{hi} cells relative to a DEAB-inhibited control (not depicted); calculation of relative Aldefluor activity is shown. Note the variation in Aldefluor activity in unconditioned MoDCs between different experiments. **B:** Summary data of median proportion of Aldefluor^{hi} unconditioned and CM-treated MoDCs + SD (n=30); p-value derives from a paired t-test. **C:** Summary data depicting relative Aldefluor activity of individual CM-treated MoDCs; HC is healthy control, NI is non-inflamed, I is inflamed; ● = HC, ▲ = CD, ■ = UC, filled and empty shapes depict CM derived from colonic and ileal biopsies, respectively; bars indicate median values.

cultured in serum-free medium (X-VIVO 10) in the presence or absence of *all-trans* retinal (100 nM) for 4 hours and RA was measured in the supernatants directly by high performance liquid chromatography with tandem mass spectrometry (LC/MS/MS). RA was detected in the supernatant of non-conditioned MoDCs cultured in the presence of retinal (mean concentration 0.32 pmol/ml). However, a significantly reduced

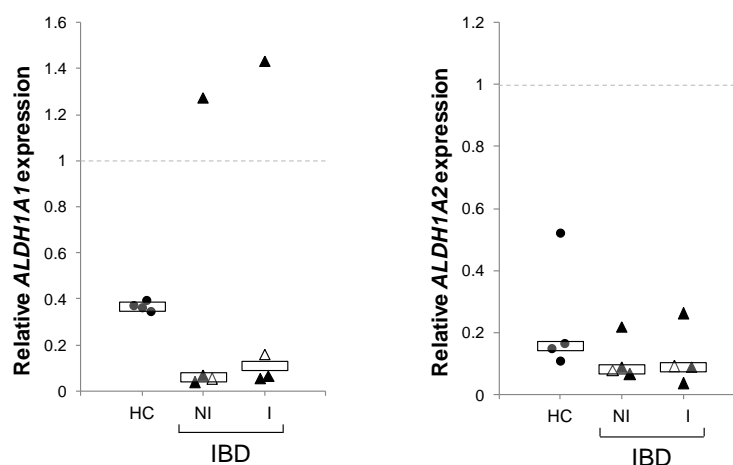


Figure 6.3: Human intestinal biopsy-derived CM suppresses *ALDH1A2* expression in MoDCs. CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) in the presence or absence of intestinal biopsy derived-CM and *ALDH1A* expression determined by real-time PCR on day 7. *ALDH1A1* and *ALDH1A2* expression in CM-treated MoDCs, normalized to *GAPDH* using $2^{-\Delta C_t}$ then expressed relative to unconditioned MoDCs. HC is healthy control (n=4), NI is non-inflamed (n=4), I is inflamed (n=4); ● = HC, ▲ = CD, ■ = UC, filled and empty shapes depict CM derived from colonic and ileal biopsies, respectively; bars indicate median values.

concentration of RA was detected in the supernatant of CM-treated MoDCs (mean concentration 0.17 pmol/ml; Figure 6.4), consistent with reduced RALDH activity in these cells. Notably, RA was undetectable in supernatants in absence of exogenous retinal. RA was detected in medium only control wells at approximately 0.1 pmol/ml (0.1 nM) in the presence of retinal (Figure 6.4), but was below the limit of detection without retinal. This indicates that a small fraction of retinal spontaneously oxidises to retinoic acid in culture or else the commercial preparation contains trace amounts of RA.

Overall, these data indicate that the human intestinal mucosa contains soluble mediators which act as negative regulators of RALDH activity in MoDCs. Contrary to expectation, the enhanced RALDH activity of CD103⁺ DCs and CD14⁺ MΦ in IBD was not observed in this system.

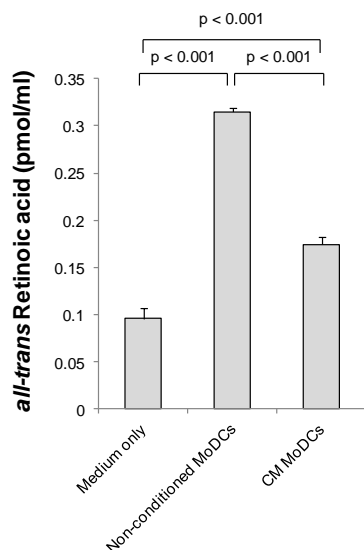


Figure 6.4: Human intestinal biopsy CM suppresses ability of MoDCs to generate RA in the presence of retinal. CD14⁺ monocytes were cultured with GM-CSF and IL-4 (100 ng/ml) in the presence or absence of intestinal biopsy-derived CM, then cultured on day 7 for 4 hours in the presence of retinal (100 nM); RA was quantified in supernatants by HPLC with MS/MS in the lab of Maureen Kane, University of Maryland, Baltimore. Cultures performed in triplicate, mean values displayed + SD; p-values derive from One Way ANOVA, with pair-wise comparisons by Tukey Test.

As discussed in Chapters 2 and 5, GM-CSF is a potent inducer of RALDH activity in human CD14⁺ blood monocytes. A possible mechanism of the suppression of RALDH activity by CM was therefore modification of GM-CSF signalling in these cells. Interaction between GM-CSF and its receptor leads, through a series of intermediate steps, to the phosphorylation of tyrosine residues on signal transducers and activators of transcription (STAT)-5 (Mui et al., 1995). STAT5 is then directed into the nucleus where it influences transcription of a wide range of genes. An experiment was therefore performed to determine if GM-CSF induced reduced levels of STAT5 phosphorylation in monocytes during MoDC differentiation cultures when in the presence of biopsy CM. Peak pSTAT5 MFI was observed at 1 hour and was reduced at both 4 and 24 hours of culture (Figure 6.5). However, no differences in pSTAT5 MFI were apparent between untreated and CM-treated MoDCs. This indicates that early GM-CSF signalling events are not disturbed by the presence of CM, and that suppression of RALDH activity occurs either downstream of this point or is independent of the GM-CSF/STAT5 signaling pathway.

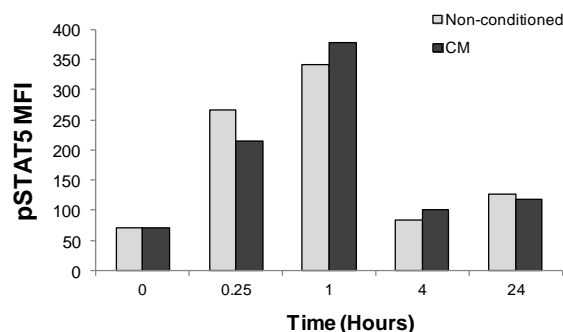


Figure 6.5: STAT5 phosphorylation in MoDCs by GM-CSF is unaltered in the presence of intestinal biopsy-derived CM. CD14⁺ monocytes were cultured with GM-CSF and IL-4 (100 ng/ml) in the presence or absence of intestinal biopsy-derived CM and were fixed at time points shown in PFA (4 %) and permeabilised in methanol (70 %). pSTAT5 MFI was determined by intracellular flow cytometry.

6:4.2 Prostaglandin E₂ contributes to suppression of RALDH activity by intestinal biopsy CM

During the course of these experiments it was reported that PGE₂ acts as a negative regulator of RALDH activity in both human and murine MoDCs (Stock et al., 2011), and it was suggested that PGE₂ generation in tissues such as the skin inhibit RALDH expression and DC imprinting of gut tropism at these sites. Stock *et al* did not examine the role of PGE₂ in the human intestine but their work suggested it was a potential mediator present within intestinal biopsy CM responsible for the suppression of RALDH activity observed in our system. In order to address this, MoDCs were grown in the presence or absence of the PGE₂ receptor antagonist AH6809, which targets the E-prostanoid (EP)₁ and EP₂ receptors (Jones et al., 2009). As expected, there was suppression of Aldefluor activity within MoDCs treated with intestinal biopsy CM, consistent with a loss of RALDH activity. However, when MoDCs were cultured with both CM and AH6809, there was partial restoration of Aldefluor activity (Figure 6.6A),

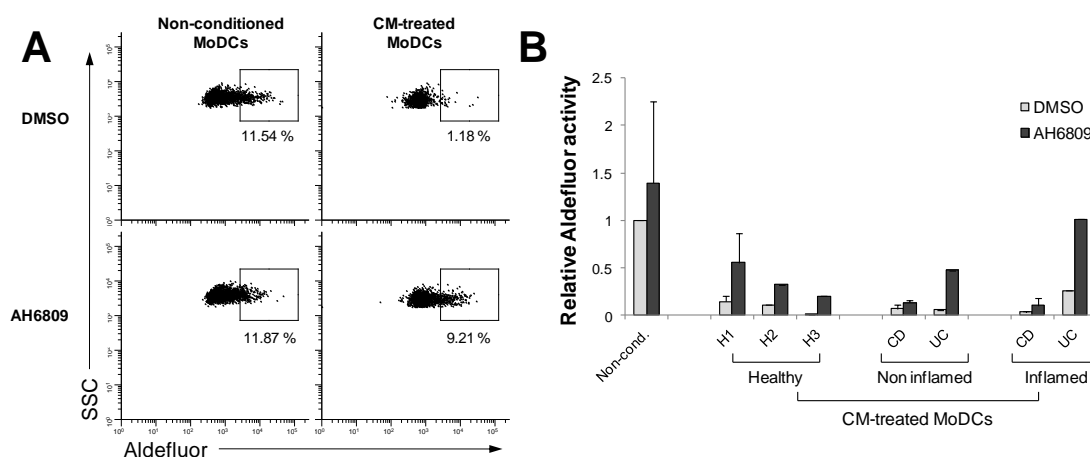


Figure 6.6: PGE₂ in human intestinal biopsy-derived CM is partially responsible for suppressed RALDH activity in CM-treated MoDCs. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and either the PGE₂ EP₁/EP₂ receptor antagonist AH6809 (10 μ M) or DMSO (vehicle control). Aldefluor analysis was performed on day 7; gate defines Aldefluor^{hi} cells relative to a DEAB-inhibited control (not depicted). **B:** Summary data of 3 independent experiments; mean Aldefluor activity relative to DMSO control depicted + SD where replicate data for a particular CM exists. Non-cond. refers to non-conditioned MoDCs.

indicating that PGE₂ signalling is involved in the suppression of RALDH activity in this system. AH6809 was able to partially restore Aldefluor activity suppressed by a range of CM, including those derived from healthy control and IBD biopsies (Figure 6.6B). This suggests that PGE₂ is present and is able to influence RALDH activity within DCs in the context of healthy and IBD intestinal mucosa. Overall, AH6809 had no significant effect on the Aldefluor activity of non-conditioned MoDCs (Figures 6.6A and B). In one of three independent experiments however, the Aldefluor activity of non-conditioned MoDCs was increased in the presence of AH6809. This indicates that in this particular experiment, inhibition of endogenous PGE₂ as opposed to intestinal biopsy-derived PGE₂ cannot be excluded as the mechanism behind the partial restoration of Aldefluor activity in CM-treated MoDCs cultured with AH6809.

Assuming a role for PGE₂ in the downregulation of RALDH activity by CM, it was unclear whether PGE₂ was present in CM itself, or whether production of PGE₂ was induced within MoDCs by factors present in CM. To address this, MoDCs were grown in the presence of CM and indomethacin, an inhibitor of cyclooxygenase (COX)-1 and COX-2 which generate the PGE₂ precursor PGH₂ from arachidonic acid (Nakata et al., 1981). Indomethacin did not restore Aldefluor activity suppressed by a healthy control, non-inflamed or inflamed IBD biopsy-derived CM (Figures 6.7A and B). This strongly suggests that PGE₂ present within the CM itself rather than induced PGE₂ production is partially responsible for suppression of RALDH activity of MoDCs in this system.

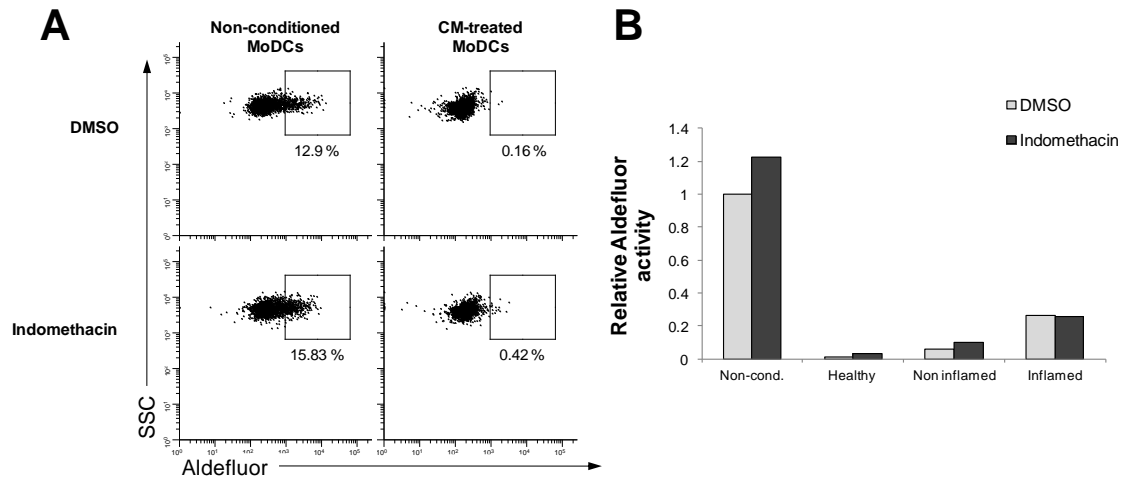


Figure 6.7: PGE₂ production induced by human intestinal biopsy-derived CM is not responsible for suppressed RALDH activity in CM-treated MoDCs. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and either the COX inhibitor indomethacin (1 μ M) or DMSO (vehicle control). Aldefluor analysis was performed on day 7; gate defines Aldefluor^{hi} cells relative to a DEAB-inhibited control (not depicted). **B:** Data from a single experiment in which 3 independent CM were tested; mean Aldefluor activity relative to DMSO control depicted.

6:4.3 Inflamed IBD biopsy CM significantly alters the differentiation of MoDCs

It has been observed elsewhere that inclusion of PGE₂ with CD14⁺ monocyte and GM-CSF/IL-4 cultures significantly alters the phenotype of human MoDCs (Kalinski et al., 1997). This includes a failure to both downregulate CD14 and to upregulate CD1a, an MHC class I-like molecule involved in presentation of endogenous and pathogen-derived lipid antigens (Brigl and Brenner, 2004). Based on PGE₂-mediated suppression of RALDH activity in CM-treated MoDCs, it was therefore hypothesized that surface phenotype would also be modified. Indeed, MoDCs grown in the presence of intestinal biopsy CM displayed increased retention of CD14, with reduced upregulation of CD1a (Figure 6.8A). Furthermore, MoDCs treated with CM derived from both non-inflamed and inflamed biopsies from IBD patients had significantly reduced expression of CD1a compared with CM derived from healthy controls (Figure 6.8B). There was a further trend in which inflamed CM-treated MoDCs had reduced CD1a expression compared with non-inflamed CM-treated MoDCs, although this did not reach statistical significance (Figure 6.8B). In addition, inflamed CM-treated MoDCs had significantly elevated expression of CD14 compared with both non-inflamed and healthy control CM-treated MoDCs (Figure 6.8C). These data indicate that factors present within intestinal biopsy CM influence MoDC differentiation in a manner consistent with the published effects of PGE₂.

6:4.4 Inflamed IBD biopsy CM enhances ability of MoDCs to induce $\alpha_4\beta_7$ expression in naive CD4⁺ T cells

Based on the presence of RALDH activity within non-conditioned MoDCs, and the demonstrated ability to produce measurable levels of RA in the presence of exogenous

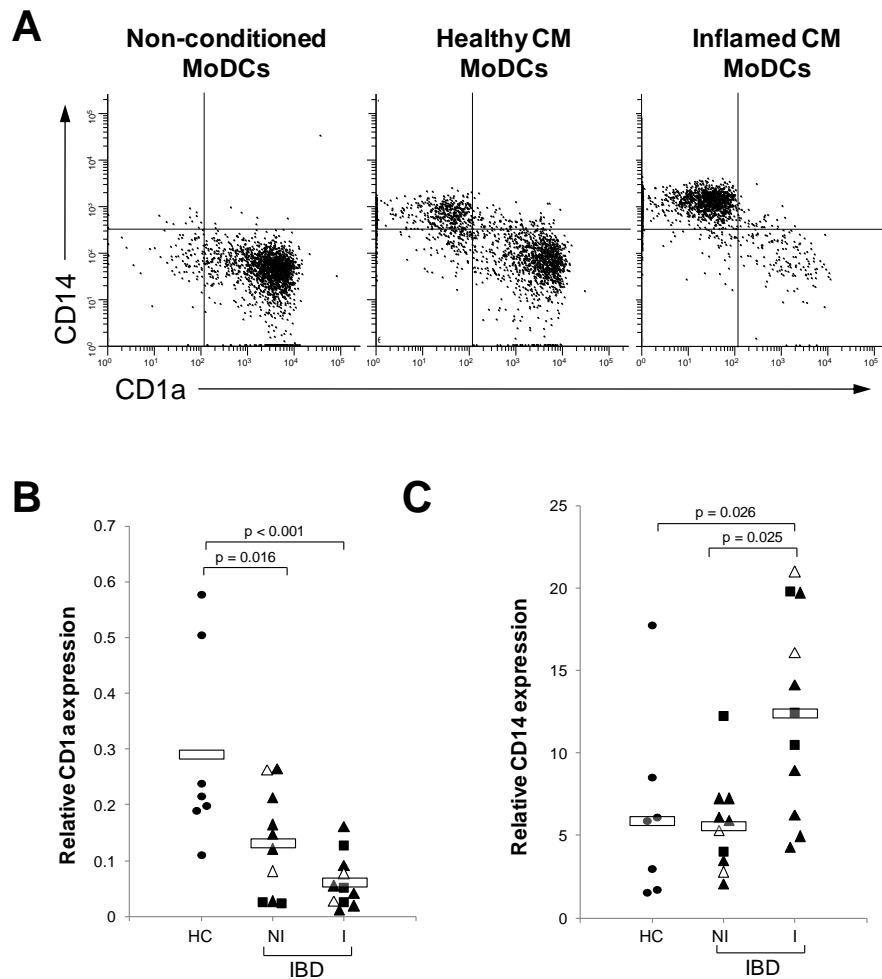


Figure 6.8: Inflamed IBD biopsy-derived CM significantly alters the differentiation of MoDCs. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and surface phenotype was analyzed by flow cytometry on day 7; representative data. **B:** Summary data of relative CD1a expression based on ratio of CD1a MFI for CM-treated MoDCs to non-conditioned MoDCs in parallel culture. **C:** Summary data of relative CD14 expression based on ratio of CD14MFI for CM-treated MoDCs to non-conditioned MoDCs in parallel culture. Each symbol represents an individual CM-treated MoDC; HC (n=7) is healthy control, NI (n=10) is non inflamed IBD and I (n=11) is inflamed IBD biopsy-derived CM; ● = Healthy, ▲ = CD, ■ = UC; filled symbols are colonic and empty symbols are ileal tissue samples; bars represent mean values in **B** and median values in **C**; p-values derived from one way ANOVA with pair-wise comparison by Tukey test; statistical analysis of **C** follows log₁₀ transformation.

retinal, it was presumed that these cells would induce RA-dependent induction of $\alpha_4\beta_7$ and CCR9 expression during the activation of naive $CD4^+$ T cells. Indeed, following 5 days co-culture of MoDCs and CFSE-labelled naive $CD4^+$ T cells, a significant fraction of proliferating CFSE^{lo} $CD4^+$ T cells were β_7^+ compared with isotype control-labelled cells (Figures 6.9A and B). These experiments were performed using a monoclonal antibody to β_7 alone as opposed to $\alpha_4\beta_7$ as the necessary antibody (Act-1) was not available at this point. However, subsequent experiments confirmed a close association between β_7 and $\alpha_4\beta_7$ expression on proliferating $CD4^+$ T cells when stimulated by MoDCs (Figures 6.10A and B), demonstrating that β_7 is sufficient as a surrogate marker for $\alpha_4\beta_7$ expression in this context. The induction of $\alpha_4\beta_7$ by MoDCs was suppressed in the presence of the RAR α antagonist Ro41-5253 (Apfel et al., 1992), demonstrating the requirement for RAR α signalling in this induction. Furthermore, the presence of exogenous RA (10 nM) enhanced induction of $\alpha_4\beta_7$ (Figures 6.10A and B). In contrast, MoDCs did not induce CCR9 expression on proliferating $CD4^+$ T cells (Figures 6.9C and D).

Based on reduced RALDH activity and therefore a presumed reduced ability to make RA, it was hypothesized that CM-treated MoDCs would induce lower levels of $\alpha_4\beta_7$ expression on proliferating $CD4^+$ T cells compared with their non-conditioned MoDC counterparts. However, naïve $CD4^+$ T cells activated by CM-treated MoDCs displayed no defects in β_7 induction. Indeed, $CD4^+$ T cells activated by some CM-treated MoDCs expressed more β_7 than T cells activated in parallel by non-conditioned MoDCs (Figures 6.11A and B).

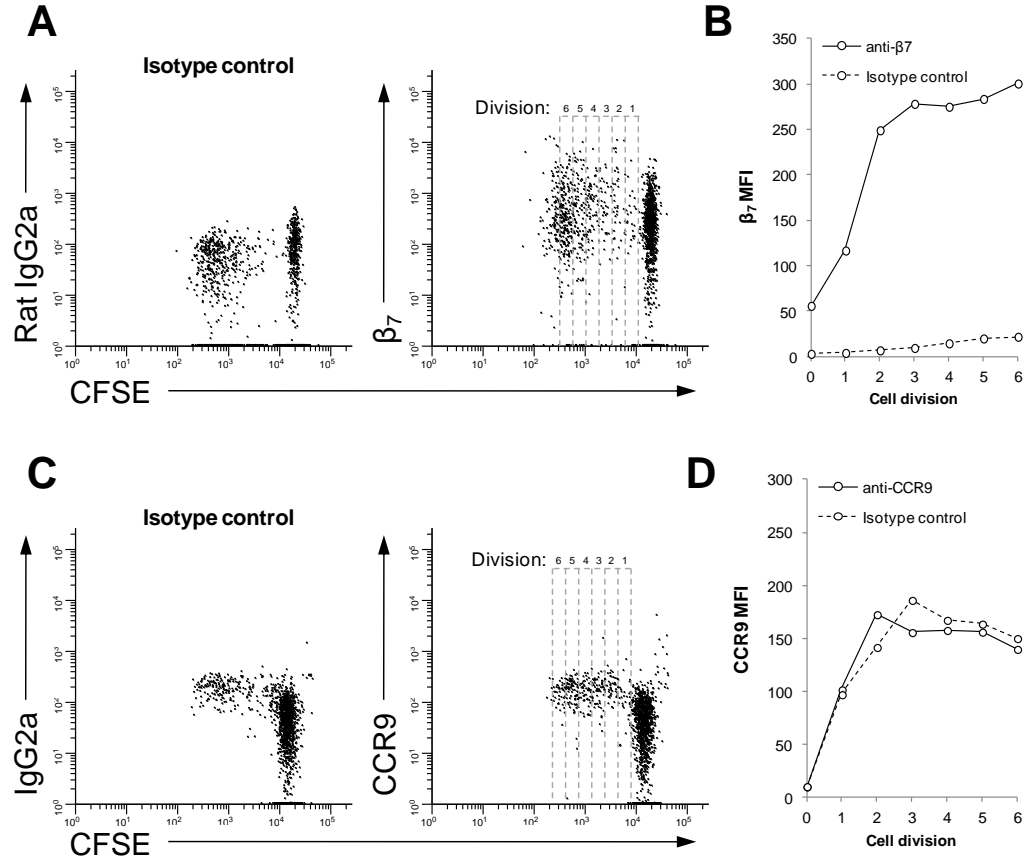


Figure 6.9: Activation by human MoDCs results in increased $\alpha_4\beta_7$ expression by allogeneic CD4⁺ T cells. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and then co-cultured with CFSE-labelled naive CD4⁺ T cells at a ratio of 1:10 and β_7 expression analysed on proliferating CFSE^{lo} cells by flow cytometry. Successive divisions were gated on the basis of CFSE dilution as indicated. **B:** β_7 MFI was determined at each cell division and compared to cells stained with the rat IgG2a isotype control antibody as indicated. **C:** In parallel cultures, CCR9 expression was determined on proliferating CD4⁺ T cells. **D:** CCR9 MFI at successive divisions compared with the mouse IgG2a isotype control antibody as in **B**.

It was observed that β_7 expression increased with successive T cell divisions (Figure 6.11 B), as has been noted elsewhere (Stagg et al., 2002, Johansson-Lindbom et al., 2003, Sheasley-O'Neill et al., 2007, Jaensson et al., 2008). The extent of T cell proliferation was therefore a potential confounding influence on the level of β_7 induction.

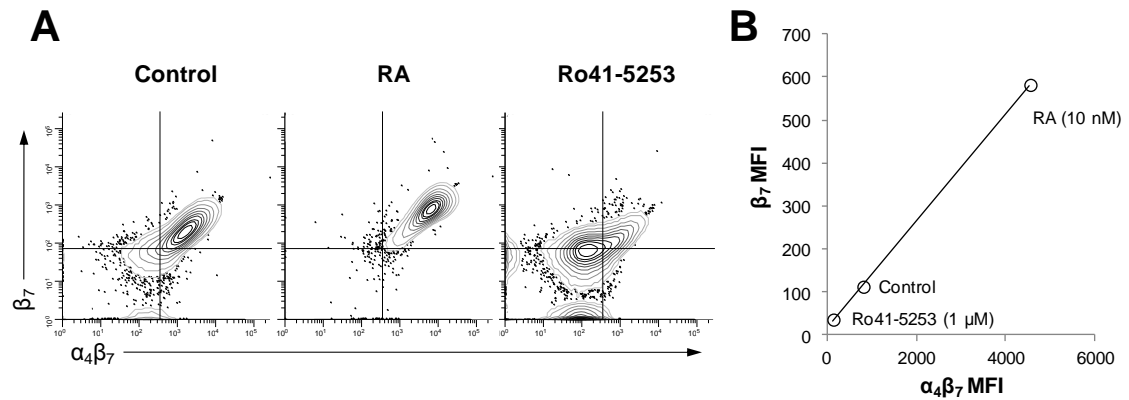


Figure 6.10: β_7 is an effective surrogate marker for $\alpha_4\beta_7$ in MoDC-naive CD4⁺ T cell co-cultures. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and then co-cultured with CFSE-labelled naive CD4⁺ T cells at a ratio of 1:10 in the presence of RA (10 nM) or Ro41-5253 (1 μ M) for 5 days, then β_7 or $\alpha_4\beta_7$ expression on CFSE^{lo} cells determined by flow cytometry. **B:** β_7 and $\alpha_4\beta_7$ MFI for the different treatment groups were correlated.

To control for this possibility, β_7 MFI was measured at a fixed point (division 6 as identified by CFSE dilution) in all experiments, and the effect of DC conditioning was determined by calculating, at this fixed point, the ratio of β_7 MFI on CD4⁺ T cells activated by CM-treated MoDCs to β_7 MFI on CD4⁺ T cells activated by non-conditioned MoDCs (Figure 6.11B). This analysis identified significantly increased induction of β_7 expression on CD4⁺ T cells activated by inflamed compared with non-inflamed IBD CM-treated MoDCs (Figure 6.11C). This represented a median fold increase in β_7 MFI over unstimulated MoDCs of 1.6 for inflamed CM-MoDCs compared with 0.9 for non-inflamed CM-treated MoDCs. This increase in β_7 was observed with MoDCs conditioned with CM derived from both CD and UC patients (Figure 6.11C). For healthy control CM-MoDCs, the median fold increase in β_7 MFI over non-conditioned MoDC was intermediate at 1.3. As expected, there was no correlation between the level of Aldefluor activity of CM-treated MoDCs and the relative induction of β_7 on proliferating

CD4⁺ T cells (Figure 6.11D). This indicates that despite equivalent suppression of

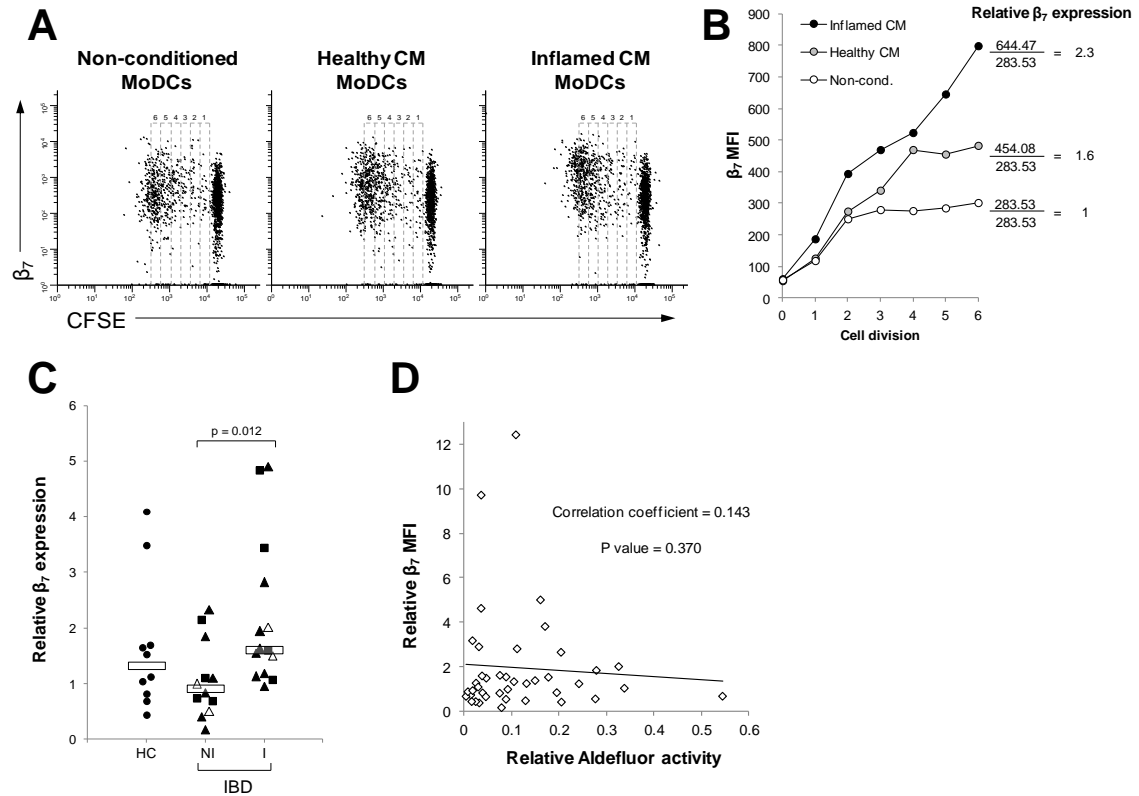


Figure 6.11: Inflamed IBD intestinal biopsy CM-treated MoDCs induce significantly greater levels of β_7 on CD4⁺ T cells than non-inflamed IBD CM-treated MoDCs. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and then co-cultured with CFSE-labelled naive CD4⁺ T cells at a ratio of 1:10 for 5 days and β_7 expression was determined on CFSE^{lo} cells by flow cytometry. **B:** β_7 MFI at successive cell divisions was determined for the different CM-treated MoDC-naive CD4⁺ T cell co-cultures; β_7 MFI at division 6 is indicated; calculation of relative β_7 expression at division 6 displayed. **C:** Summary data of relative β_7 expression of CD4⁺ T cells at division 6 following co-culture with intestinal biopsy-derived CM-treated MoDCs; HC (n=10) is healthy control, NI (n=12) is non inflamed IBD and I (n=14) is inflamed IBD biopsy-derived CM; ● = Healthy, ▲ = CD, ■ = UC; filled and empty symbols are CM derived from colonic and TI biopsies, respectively; bars represent median values; p-value derives from one way ANOVA with pair-wise comparison by Tukey test following log₁₀ transformation of data. **D:** Correlation between relative β_7 expression of CD4⁺ T cells at division 6 and relative Aldefluor activity of match intestinal biopsy CM-treated MoDCs; p-value derives from a Spearman Rank Order Correlation.

RALDH activity across the range of CM sub-groups, differences exist in their ability to influence the induction of $\alpha_4\beta_7$ by MoDCs, in particular between inflamed and non-inflamed IBD CM.

Murine E-selectin ligand induction is negatively regulated by RA (Iwata et al., 2004). To confirm the reciprocal induction of the human equivalent, cutaneous lymphocyte antigen (CLA), and $\alpha_4\beta_7$ by RA in humans, naive CD4⁺ T cells were stimulated with activation beads (coated with anti-CD2, CD3 and CD28) in the presence of various concentrations of RA and the RAR α antagonist Ro41-5253. As expected, increasing the concentration of RA to 10 nM led to greater induction of β_7 on proliferating CD4⁺ T cells which was suppressed in the presence of Ro41-5253 (Figure 6.12). Furthermore, Ro41-5253 also suppressed the induction of β_7 in the absence of exogenous RA. In contrast, increasing the concentration of RA suppressed the induction of CLA, which could be inhibited by Ro41-5253 at lower concentrations of RA (0.1 and 2 nM). In contrast with β_7 , Ro41-5253 enhanced induction of CLA in the absence of exogenous RA (Figure 6.12).

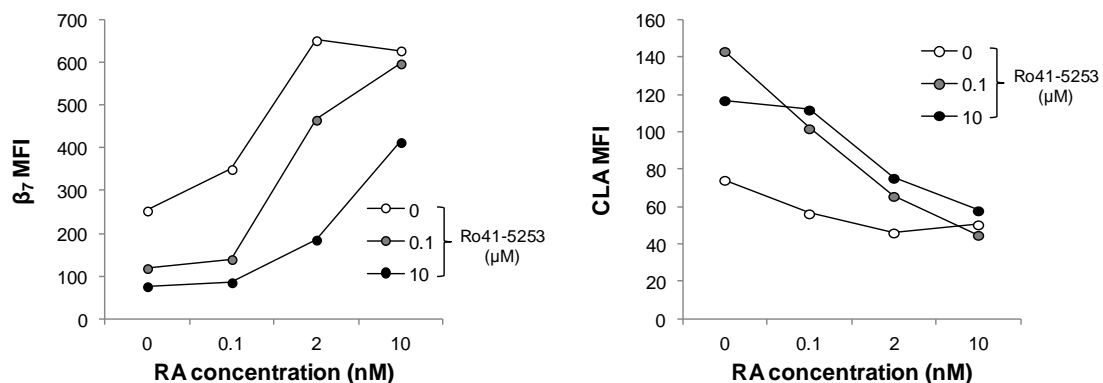


Figure 6.12: RA enhances $\alpha_4\beta_7$ but suppresses CLA expression during the activation of naive CD4⁺ T cells. A: CFSE-labelled naive CD4⁺ T cells were stimulated with microbeads coated with anti-CD2/CD3/CD28 for 5 days in varying concentrations of RA and Ro41-5253 as indicated; β_7 and CLA MFI were determined on CFSE₁₀ cells.

The induction of increased levels of $\alpha_4\beta_7$ on proliferating CD4⁺ T cells by inflamed compared with non-inflamed IBD CM-MoDCs was consistent with elevated levels of RA signalling in the inflamed IBD CM MoDC-T cell co-cultures. Based on the reciprocal regulation of $\alpha_4\beta_7$ and CLA expression on CD4⁺ T cells by RA as confirmed above, it was hypothesised that inflamed IBD CM-MoDCs would therefore induce less CLA on proliferating CD4⁺ T cells as compared with non-inflamed IBD CM-MoDCs. Non-conditioned MoDCs induced expression of CLA on a small proportion of proliferating CD4⁺ T cells (Figures 6.13A and B). The influence of CM treatment of MoDCs on induction of CLA expression by CD4⁺ T cells was variable. However, suppression of CLA was observed in a number of experiments, consistent with enhanced RA signalling (Figures 6.13C and D).

CLA expression increased with successive T cell divisions (Figure 6.13B), and so a relative CLA expression level was determined in the same manner as for β_7 to allow comparison between experiments. Overall, the pattern of CLA induction by healthy, non-inflamed and inflamed IBD CM MoDCs was reciprocal to that of β_7 . Specifically, there was a trend towards lower expression of CLA when naive CD4⁺ T cells were activated by inflamed CM-treated MoDCs than when the T cells were activated by non-inflamed IBD CM-treated MoDCs, although this did not reach statistical significance (Figure 6.13E). Levels of CLA suppression by healthy and inflamed IBD CM-treated MoDCs were similar. Overall, this suggests that soluble factors within inflamed but not non-inflamed IBD mucosa are able to enhance induction $\alpha_4\beta_7$ at the expense of CLA expression on CD4⁺ T cells by MoDCs, independently of RALDH activity.

It remained unclear as to why induction of $\alpha_4\beta_7$ on proliferating CD4⁺ T cells should be independent of RALDH activity in MoDCs. Based on the inability of MoDCs to produce detectable levels of RA in the absence of exogenous retinal (Figure 6.4), it was

hypothesized that the RALDH activity in MoDCs was unable to generate sufficient RA to influence β_7 expression. To address this possibility, MoDCs were compared directly

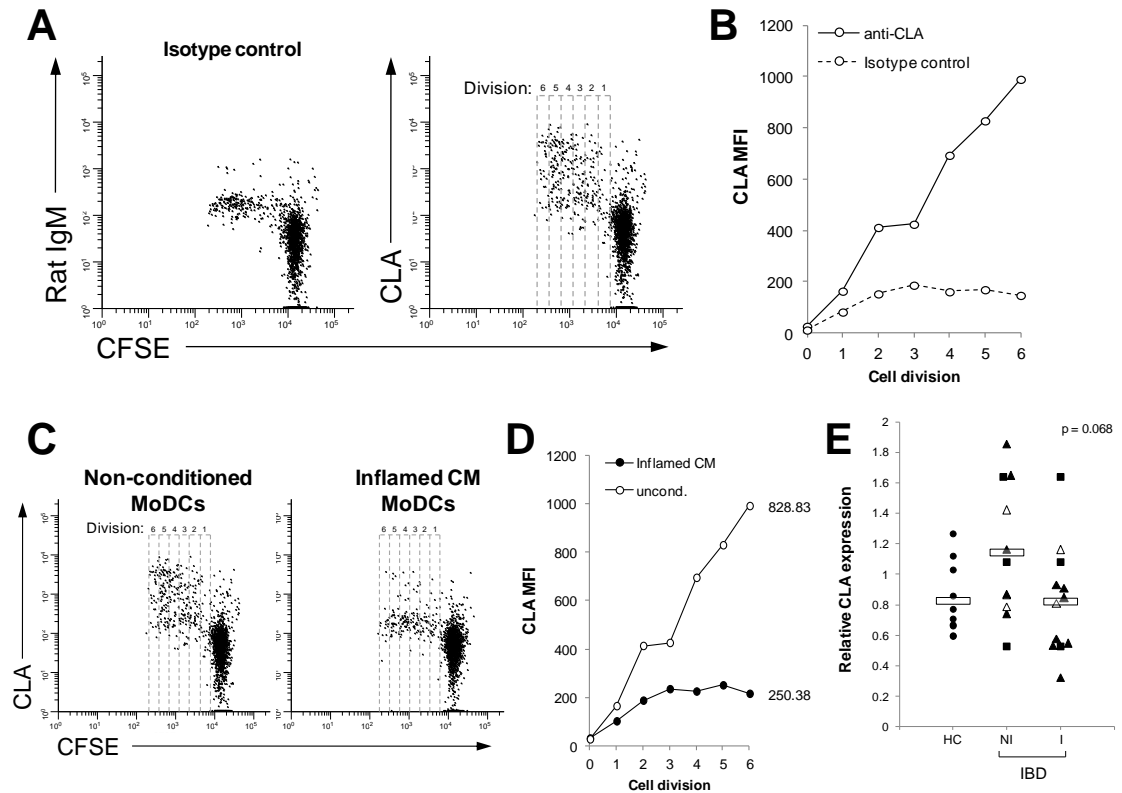


Figure 6.13: Inflamed IBD intestinal biopsy CM-treated MoDCs induce lower levels of CLA on CD4⁺ T cells than non-inflamed IBD CM-treated MoDCs. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and then co-cultured with CFSE-labelled naive CD4⁺ T cells at a ratio of 1:10 and CLA expression analysed on proliferating CFSE^{lo} cells by flow cytometry. Successive divisions were gated on the basis of CFSE dilution as indicated. **B:** CLA MFI was determined at each cell division and compared to cells stained with the rat IgM isotype control antibody as indicated. **C:** Example of differential induction of CLA on proliferating CD4⁺ T cells by non-conditioned and CM-treated MoDCs. **D:** CLA MFI on proliferating CD4⁺ T cells activated by non-conditioned and CM-treated MoDCs at successive divisions. **E:** Summary data comparing relative CLA expression (calculated as for β_7) in CM-treated MoDCs; HC (n=8) is healthy control, NI (n=10) is non inflamed IBD and I (n=12) is inflamed IBD biopsy-derived CM; ● = Healthy, ▲ = CD, ■ = UC; filled and empty symbols are CM derived from colonic and TI biopsies, respectively; bars represent median values; p-value derives from one way ANOVA.

with 'walk out' cells obtained from colonic biopsies that induce significant levels of $\alpha_4\beta_7$ on proliferating CD4⁺ T cells, as observed in Chapter 4. In a parallel co-culture experiment, 'walk out' cells induced a substantially greater level of β_7 expression on proliferating CD4⁺ T cells as compared with that induced by non-conditioned MoDCs (Figures 6.14A and B). This indicates that the level of $\alpha_4\beta_7$ induction by MoDCs is relatively low compared with intestinal APCs, and is consistent with low or absent RA production by the RALDH enzymes present in these cells. Both MoDCs and 'walk out' cells induced low levels of CLA, whilst neither induced CCR9 expression on CD4⁺ T cells (Figure 6.14A).

An alternative hypothesis to explain differential induction of β_7 expression on CD4⁺ T cells by CM-treated MoDCs independent of RALDH activity was a modified ability to oxidise retinol present in serum to retinal, the RALDH substrate. The oxidation of retinol to retinal is rate-limiting in the presence of CRBP-I for the overall production of RA (reviewed by Napoli, 2012). Equivalent levels of retinol (2.5 nmol/ml; 2.5 μ M) were detected in all CM tested by HPLC MS/MS (Figure 6.15), indicating that MoDCs were not exposed to different levels of retinol depending on the CM group. Expression of *RDH10* and *DHRS9* was determined in CM-treated MoDCs by real-time PCR to assess the influence of CM-treatment on expression of these retinol oxidising enzymes. As with *ALDH1A2* (Figure 6.3), *RDH10* expression was suppressed by healthy control, non-inflamed and inflamed IBD CM to approximately 50 % of the level observed in non-conditioned MoDCs (Figure 6.16). In contrast, *DHRS9* expression was suppressed by some but not all CM tested. Of particular interest was a trend in which *DHRS9* expression was suppressed in three of four non-inflamed CM-treated MoDCs tested, whilst both healthy and inflamed IBD CM-treated MoDCs had similar distributions of *DHRS9* expression (Figure 6.16). These levels of expression match the distribution of

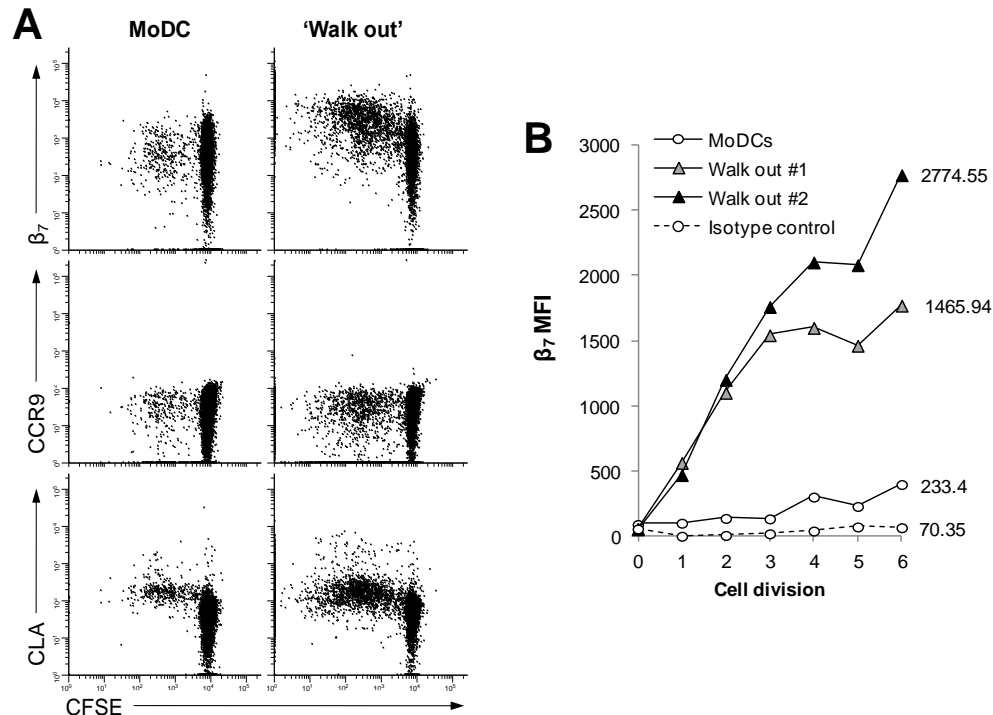


Figure 6.14: ‘Walk out’ cells derived from colonic biopsies induce substantially greater levels of $\alpha_4\beta_7$ on CD4⁺ T cells than MoDCs. **A:** CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM and then co-cultured with CFSE-labelled naive CD4⁺ T cells at a ratio of 1:10. ‘Walk out’ cells derived from colonic biopsies were also co-cultured with naive CD4⁺ T cells at a ratio of 1:4 for 5 days and expression of $\alpha_4\beta_7$, CCR9 and CLA determined on proliferating CFSE^{lo} cells by flow cytometry. **B:** β_7 MFI was determined at successive CD4⁺ T cell divisions; MFI at division 6 is indicated.

β_7 induction in CD4⁺ T cells, although differences do not reach statistical significance. Overall, these data may indicate that the ability of MoDCs to oxidise the available retinol to retinal via *DHRS9* expression is a key determinant of overall RA output and induction of $\alpha_4\beta_7$ in CD4⁺ T cells.

In addition to retinol, some CM contained detectable levels of RA (Figure 6.15). Therefore, an alternative hypothesis explaining the induction of β_7 in CD4⁺ T cells stimulated by inflamed and non-inflamed IBD CM-treated MoDCs may have related to

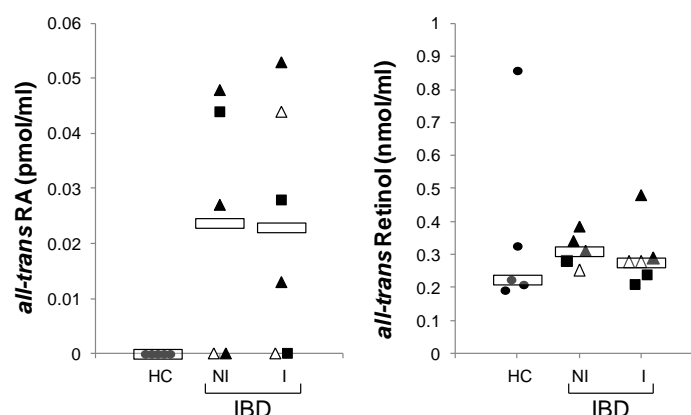


Figure 6.15: Variable concentrations of retinoids detected in intestinal biopsy-derived CM. Intestinal biopsy CM were analyzed by HPLC MS/MS for the presence of *all-trans* RA and *all-trans* retinol in the lab of Maureen Kane, University of Maryland, Baltimore; HC (n=5) is healthy control, NI (n=5) is non inflamed IBD and I (n=6) is inflamed IBD biopsy-derived CM; ● = Healthy, ▲ = CD, ■ = UC; filled and empty symbols are CM derived from colonic and TI biopsies, respectively; bars represent median values.

‘carry-over’ of RA directly from the biopsy into CM. Levels of RA in CM derived from five healthy control biopsies were too low to be reliably quantified by this approach. RA was detected in both inflamed and non-inflamed biopsy CM, at an average level of 0.02 pmol/ml (20 pM; Figure 6.15). However, there were no apparent differences between inflamed and non-inflamed RA levels. Together with the very low levels detected, this indicates that ‘carry-over’ of RA directly from the biopsies into CM is unlikely to be responsible for the differences in induction of β_7 by inflamed and non-inflamed IBD CM-treated MoDCs.

The inhibition of β_7 induction on CD4⁺ T cells by MoDCs in the presence of Ro41-5253 (Figures 6.10A and B and Figure 6.12) strongly implicated RAR α signalling in this process. The level of *RARA* expression (encoding RAR α) in proliferating CD4⁺ T cells was therefore a further potential source of differential induction of β_7 expression. To address this hypothesis, naive CD4⁺ T cells were stimulated with non-conditioned MoDCs and MoDCs treated with an inflamed CM that lead to a 2 fold increase in β_7 MFI

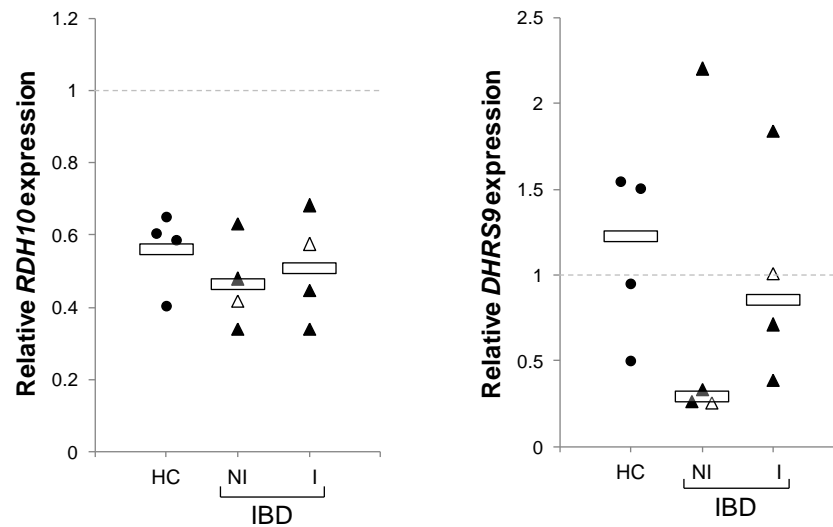


Figure 6.16: Intestinal biopsy-derived CM suppresses *RDH10* expression in MoDCs but has variable effects on *DHRS9* expression. CD14⁺ monocytes were cultured in GM-CSF and IL-4 (100 ng/ml) for 7 days in the presence or absence of intestinal biopsy CM then *RDH10* and *DHRS9* expression was determined by real-time PCR; expression normalized to *GAPDH* using $2^{-\Delta Ct}$ then expressed relative to unconditioned MoDCs; HC is healthy control (n=4), NI is non-inflamed (n=4), I is inflamed (n=4); ● = HC, ▲ = CD, ■ = UC, filled and empty shapes depict CM derived from colonic and ileal biopsies, respectively; bars indicate mean and median values for *RDH10* and *DHRS9*, respectively.

at division 6 (data not shown). Proliferating CD4⁺ T cells (CFSE^{lo}) were sorted by flow cytometry and *RARA* expression was quantified by real-time PCR. The expression of *RARA* was identical in CD4⁺ T cells stimulated by both non-conditioned MoDCs and inflamed CM-treated MoDCs (Figure 6.17). This indicates that at the level of transcription, *RARα* is not modified during CD4⁺ T cell activation by CM-treated MoDCs. Modulation of *RARα* expression is therefore unlikely to explain differential induction of β_7 expression on CD4⁺ T cells by CM-treated MoDCs.

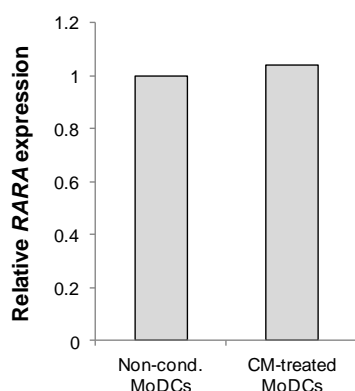


Figure 6.17: Expression of *RARA* is similar in CD4⁺ T cells stimulated by non-conditioned and CM-treated MoDCs. CFSE-labelled naive CD4⁺ T cells were co-cultured with non-conditioned MoDCs or MoDCs differentiated in the presence of a CM which had enhanced the ability of MoDCs to induce β_7 by 2 fold in other experiments; proliferating CFSE^{lo} cells were sorted by flow cytometry and *RARA* expression was determined by real-time PCR relative to *GAPDH* by the $2^{-\Delta Ct}$ method and normalized to expression in CD4⁺ T cells stimulated by non-conditioned MoDCs.

As previously stated, expression of β_7 was noted at a particular division to control for the effect of additional divisions boosting perceived levels. However, a high overall level of proliferation may indicate strong signalling through the T cell receptor which itself could influence $\alpha_4\beta_7$ induction (Svensson et al., 2008). Indeed, a positive correlation was detected between the overall level of T cell proliferation and the relative expression of β_7 at division 6 (Figure 6.18A). Whilst this correlation was highly statistically significant, the coefficient of correlation was low at 0.432, indicating a weak correlation. Systematic changes in the overall level of proliferation were not likely to have influenced the level of β_7 induction however as no statistically significant differences were observed in proliferation levels across healthy, inflamed and non-inflamed IBD CM-treated MoDC T cell stimulations (Figure 6.18B). Overall therefore, human MoDCs generated in the presence of inflamed intestinal biopsy-derived CM display an increased ability to induce $\alpha_4\beta_7$ expression on allogeneic CD4⁺ T cells compared with non-inflamed IBD biopsy-derived CM-treated MoDCs. This effect is RALDH-independent, but the mechanism remains undefined.

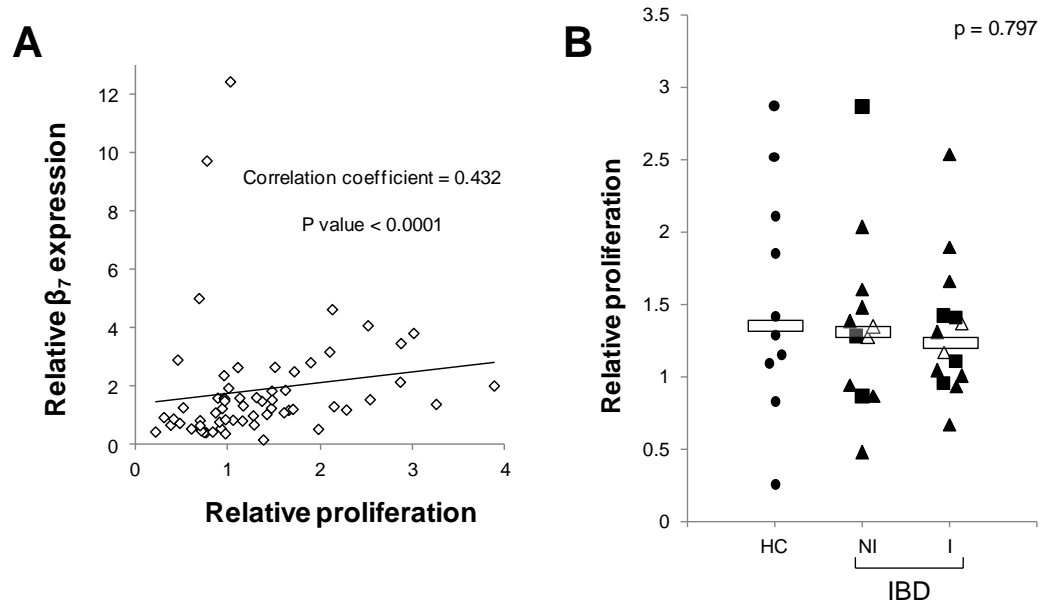


Figure 6.18: No system differences observed in the ability of CM-treated MoDCs to induce proliferation of naive CD4⁺ T cells. **A:** Relative β_7 expression of CD4⁺ T cells at division 6 was correlated against the relative level of proliferation in the co-culture (% CFSE^{lo} cells in CM-MoDC-T cell co-cultures relative to %CFSE^{lo} cells in non-conditioned MoDC-T cell co-cultures); p-value derives from Spearman Rank Order Correlation. **B:** Relative level of CD4⁺ T cell proliferation following activation with CM-treated MoDCs, calculated as in **A**; HC is healthy control (n=10), NI is non-inflamed (n=12), I is inflamed (n=14); ● = HC, ▲ = CD, ■ = UC, filled and empty shapes depict CM derived from colonic and ileal biopsies, respectively; bars indicate mean values; p-value derives from a one way ANOVA.

6:5 Discussion

This chapter aimed to understand how soluble mediators within human intestinal mucosa influence the RALDH activity of DCs. It was observed that such factors exerted negative regulation on RALDH activity and *ALDH1A2* expression in MoDCs when included from the start of culture, and that this effect was apparent regardless of whether the original biopsies were derived from healthy control or IBD patients. These findings indicate that some DCs in the intestinal mucosa may operate in an environment where there is default suppression of GM-CSF-induced RALDH activity. Whether this suppression also applies to other populations of DC or other pathways of RALDH induction within the human intestine remains to be determined.

The identity of one major source of negative RALDH regulation in intestinal biopsy-derived CM was PGE₂, which was identified by others during the course of this work as suppressive for DC RALDH activity in both mice and humans (Stock et al., 2011). Accordingly, CM-treated MoDCs grown in the presence of the PGE₂ receptor antagonist AH6809 displayed at least partial restoration of Aldefluor activity, consistent with restored RALDH activity. AH6809 is a selective antagonist for EP₁ and EP₂ receptors, whilst at higher concentrations (300 µM) can antagonise PGD₂ and thromboxane (TBX) receptors (Jones et al., 2009, Keery and Lumley, 1988). As neither PGD₂, TBX nor the EP₁-selective agonist (D1-004) suppress RALDH activity in human MoDCs (Stock et al., 2011), it is therefore likely that AH6809 restores RALDH activity in this system through antagonism of PGE₂-EP₂ interactions. Furthermore, inhibition of COX enzymes during MoDC differentiation with indomethacin had no restorative effect on RALDH activity, strongly suggesting that PGE₂ derived from CM and not induction of PGE₂ synthesis in MoDCs was responsible for this effect. It is unclear whether a

greater concentration of AH6809 would lead to further restoration of RALDH activity, or whether additional negative factors are present in CM.

This inhibitory effect of CM and the role of PGE₂ could be explored further by analysing *ALDH1A* expression by real-time PCR following AH6809-mediated restoration of Aldefluor activity in CM-treated MoDCs. In addition, the ability of these cells to convert retinal to RA could be analysed by HPLC MS/MS as used later in this Chapter. This would confirm that restored Aldefluor activity closely matches a restoration of RALDH activity.

The inflamed intestinal mucosa of UC patients contains a higher concentration of PGE₂ than is found in the mucosa of healthy controls (Sharon et al., 1978, Carty et al., 2000). As noted earlier, COX-1 is constitutively expressed within the human intestinal epithelium, whilst COX-2 expression is induced in the epithelium in IBD in response to inflammatory mediators such as IL-1 β , TNF α and LPS (Stenson, 2007). Furthermore, expression of microsomal PGE synthase-1 (mPGES-1), which specifically generates PGE₂ from PGH₂, is enhanced in inflamed colonic epithelium of both CD and UC patients (Subbaramaiah et al., 2004). This coincides with reduced expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which metabolises PGE₂ in IBD patients (Subbaramaiah et al., 2004). It would therefore be anticipated that if PGE₂ is the principal agent of negative regulation of RALDH, there should be a greater degree of RALDH suppression in MoDCs by CM derived from inflamed IBD compared with healthy control biopsies. There are multiple possibilities as to why this is not the case. The RALDH activity of MoDCs may be particularly sensitive to suppression, in which case the system lacks the sensitivity to detect more subtle differences in PGE₂ concentration present in CM. There is evidence in mice of constitutive COX-2 expression and production of PGE₂ in small intestinal stromal cells (Newberry et al.,

1999, Newberry et al., 2001) which is not influenced by a range of inflammatory stimuli including LPS, TNF α , IL-1 β , IFN γ and IL-12. Assuming this is present in the human intestine, and alongside known constitutive COX-1 activity (Stenson, 2007), PGE₂ in CM derived from healthy control biopsies may be sufficient to suppress a large proportion of MoDC RALDH activity.

An alternative explanation may relate to the way in which CM is generated. PGE₂ is known to mediate epithelial responses to injury, including the enhancement of epithelial proliferation (Stenson, 2007). COX enzyme activity may be induced in intestinal biopsies during the 6 hour culture period in which CM is generated, in response to detection of 'epithelial injury' associated with biopsy collection. This would boost the levels of PGE₂ in the CM and may therefore mask background levels of PGE₂ that are found within the intestinal mucosa across health and disease. This possibility could be addressed in future work by blocking PGE₂ synthesis during the 6 hour culture period with the COX inhibitor indomethacin. This would prevent the generation of PGE₂ in response to 'epithelial injury', allowing only background PGE₂ to influence RALDH activity in MoDCs. This would provide an important insight into how PGE₂ influences RALDH activity in human intestinal DCs in health and IBD. This work could be further developed by determining whether the PGE₂ in CM is epithelial-derived. This could be done by generating CM from biopsies in which the epithelium has been removed by EDTA treatment and in parallel with biopsies in which the epithelium is intact.

The proposed mechanism by which PGE₂ suppresses RALDH activity in MoDCs is based on elevation of intracellular levels of cyclic adenosine monophosphate (cAMP) (Stock et al., 2011). This effect is mediated specifically by the E-prostanoid (EP)-2 receptor, and stimulates inducible cAMP early repressor (ICER) which directly inhibits transcription of *Aldh1a2*. A direct effect on *ALDH1A2* expression such as this would

operate independently of GM-CSF signalling which may explain why levels of p-STAT5 were similar in MoDC cultures irrespective of exposure to CM. Stock *et al.* also demonstrated that MLN DCs in mice with high RALDH activity expressed very low levels of EP₂, whereas DCs in the same site with low RALDH activity expressed far higher levels of EP₂. This indicates that sensitivity to PGE₂ via EP₂ expression may be a key determinant of whether DCs are likely to respond to PGE₂ in this way. This raises an important question of the extent to which MoDCs will behave like other intestinal DC populations, including CD103⁺ DCs, in response to these soluble mediators. It is interesting to note that the distribution of relative Aldefluor activities across MoDCs treated with healthy, non-inflamed and inflamed IBD CM resembles more closely the Aldefluor activities of *ex vivo* CD103⁻ rather than CD103⁺ DCs derived from equivalent contexts as discussed in Chapter 4. It would be interesting to compare EP₂ expression across the different human intestinal DC subsets to determine whether differences in the ability to respond to PGE₂ could explain the distinct influence of inflammation on the RALDH activity of CD103⁺ and CD103⁻ DCs. One hypothesis is that CD103⁺ DCs express lower EP₂ levels and signals that promote RALDH activity in IBD encounter less resistance from PGE₂ signalling.

Beyond negative regulation of RALDH activity in CM-treated MoDCs, PGE₂ may have also contributed to the modified surface phenotype of these cells. The retention of CD14 expression and the failure to upregulate CD1a is consistent with effects observed with PGE₂ treatment elsewhere (Kalinski *et al.*, 1997). This altered differentiation could be interpreted as a failure of monocytes to differentiate. However, CM-treated MoDCs displayed no defect in their ability to induce T cell proliferation which suggests that key DC properties remain intact. This is also consistent with PGE₂-treated MoDCs (Kalinski *et al.*, 1997). The significantly increased retention of CD14 and failure to upregulate

CD1a in inflamed CM compared with non-inflamed and healthy control CM-treated MoDCs is also consistent with a greater concentration of PGE₂ present in the CM. This would support the argument that the RALDH activity of MoDCs is too sensitive to suppression to detect subtle increases in PGE₂ concentration, whereas CD14 expression for instance is not. It would therefore be informative to analyse the surface phenotype of MoDCs grown in the presence of intestinal biopsy-derived CM in the presence or absence of AH6809.

Beyond PGE₂, other factors potentially present in CM have been shown to influence MoDC differentiation in a similar manner. Inclusion of TLR ligands including LPS, which is likely to be present in CM, during MoDC differentiation leads to retention of CD14 and failure to upregulate CD1a (Bartz et al., 2006). The proposed mechanism is an upregulation of suppressor of cytokine signalling (SOCS)-1 and -3, which suppresses the ability of GM-CSF to signal through p-STAT5. There are a few key differences to note here however. MoDCs differentiated in the presence of LPS are poor activators of allogeneic naive CD4⁺ T cells compared with untreated MoDCs (Bartz et al., 2006). In contrast, CM-treated MoDCs displayed no defect in their ability to induce T cell proliferation; with a 1.3 fold mean increase over proliferation induced by non-conditioned MoDCs. The ability of CM-treated MoDCs to stimulate naive CD4⁺ T cells is consistent with differentiation into a viable MoDC phenotype. Furthermore, in a preliminary experiment there was very little difference observed in the MFI of p-STAT5 in CM-treated and non-conditioned MoDCs. This suggests that whilst the overall CD14⁺CD1a⁻ phenotype appears to be similar, inhibition of GM-CSF signalling by early induction of SOCS1 or SOCS3 is not responsible here.

The upregulation of CD1a occurs throughout MoDC differentiation, and may be blocked at any point by addition of an inflammatory cytokine cocktail (Gogolak et al., 2007). This

cocktail includes PGE₂ alongside TNF α , IL-1 β , IL-6. The relative contributions of each cytokine to this overall effect is however unclear. Intestinal biopsy-derived CM are likely to contain a wide range of mediators beyond PGE₂, and so further experiments are required to determine whether PGE₂ is the primary mediator of modified CD14/CD1a expression by CM-treated MoDCs. This would include determining if AH6809 can at least partially restore a 'non-conditioned' MoDC phenotype to these cells, as observed with RALDH activity. Importantly, MoDCs differentiated in the presence of intestinal biopsy-derived CM and neutralising antibodies to TNF α or IL-6 displayed severely reduced cell viability, precluding this approach as a means of identifying a role for these cytokines in the suppression of RALDH activity in MoDCs. This suggests that low levels of these cytokines are required for the survival and differentiation of MoDCs *in vitro*.

MoDCs grown in the presence of inflamed IBD intestinal biopsy-derived CM possessed a significantly enhanced ability to imprint naive CD4⁺ T cells with $\alpha_4\beta_7$ expression as compared with the ability of non-inflamed IBD CM-treated MoDCs to induce $\alpha_4\beta_7$. This would be predicted to lead *in vivo* to an increased proportion of CD4⁺ T cells with a 'gut-homing' phenotype when activated by DCs migrating into lymphoid tissue from inflamed mucosal areas. This would produce superior interactions between $\alpha_4\beta_7$ and endothelial MAdCAM-1 throughout the gastrointestinal tract, and would in turn bias intestinal T cell populations in favour of antigen specificities associated with the inflamed mucosa. This process could be associated with the dissemination of inflammation to distinct sites in the intestinal mucosa. CD103⁺ DCs sorted from MLNs draining the inflamed TI of CD patients induce similar levels of $\alpha_4\beta_7$ and CCR9 expression on naive CD8⁺ T cells as compared with those derived from healthy controls (Jaensson et al., 2008). However this work did not address how DC subsets migrating from the inflamed and non-

inflamed intestinal mucosa may differ in their ability to induce these 'gut-homing' markers.

The mechanism of enhanced induction of $\alpha_4\beta_7$ on CD4⁺ T cells by inflamed compared with non-inflamed IBD CM-treated MoDCs is independent of effects on RALDH activity, but ultimately remains undefined. Irrespective of the underlying mechanism, this finding is important as it may suggest that factors within the inflamed human intestinal mucosa influence the ability of DCs to induce RA signalling in T cells during activation, with or without the presence of additional RA. The loss of RALDH activity in MoDCs following differentiation in the presence of intestinal biopsy CM and the maintained ability to induce $\alpha_4\beta_7$ on CD4⁺ T cells implies that despite ALDH1A expression and the presence of RALDH activity (Chapter 3), non-conditioned MoDCs do not produce sufficient RA to influence $\alpha_4\beta_7$ expression. Indeed, RA was only detectable in the supernatant of non-conditioned MoDCs following the addition of exogenous retinal suggesting that the availability of RALDH substrate may be limiting for RA generation. Supernatant of CM-treated MoDCs cultured in the presence of exogenous retinal contained a significantly reduced concentration of RA, consistent with suppression of RALDH activity. Induction of $\alpha_4\beta_7$ on CD4⁺ T activated by MoDC was nonetheless dependent upon RA, most likely derived from the cell culture serum. Indeed, anti-CD2/CD3/CD28 bead-activated CD4⁺ T cells also upregulated $\alpha_4\beta_7$ and this effect was inhibited in the presence of Ro41-5253. It has been estimated that approximately 0.2 – 0.5 nM (0.2 – 0.5 pmoles/ml) RA is present in complete medium containing 10 % FCS (Kang et al., 2011). In the experiments reported in this study, no CM contained more than 0.01 pmoles/ml RA (the limit of detection) but it should be noted that RA is unstable (Napoli, 2012) and so may be degraded during CM generation. Addition of exogenous RA to T cell stimulation cultures did lead to further increases in $\alpha_4\beta_7$, confirming the potential for further integrin

expression when concentrations of the retinoid are increased. Ideally, CM-MoDC and naive CD4⁺ T cell co-cultures should be carried out in serum-free medium to remove the RA contribution from serum. However, this was not possible as naive CD4⁺ T cells proliferated poorly where these co-cultures were attempted.

The oxidation of retinol to retinal is rate limiting in the production of RA in the presence of CRBP-I (reviewed by Napoli, 2012). This step may therefore provide a key control point in the output of RA by MoDCs (Napoli, 1986). Indeed, RA generation by MoDCs was only detectable when the cultures were supplemented with exogenous retinal. Expression by MoDCs of a number of enzymes capable of oxidising retinal to retinal was confirmed. The expression of *RDH10* by MoDCs was suppressed by all CM tested, indicating similar regulation of transcription as *ALDH1A2* which may be mediated by PGE₂ directly. In contrast, *DHRS9* expression was more varied following CM treatment, with some CM-treated MoDCs displaying enhanced expression of *DHRS9* compared with non-conditioned MoDCs. This could indicate a differential relationship between *DHRS9* expression and PGE₂ as compared with *RDH10* or *ALDH1A2*. It was observed in two samples in Chapter 4 that *RDH10* expression was enhanced in human intestinal CD103⁺ compared with CD103⁻ DCs. This could enhance the ability of CD103⁺ DCs to produce RA through enhanced production of retinal. A similar system may therefore operate in CM-treated MoDCs in relation to expression of *DHRS9*. If the rate of retinal oxidation is faster than retinol oxidation, then reduced RALDH activity of CM-treated MoDCs would not significantly reduce overall RA output as this will be closely associated with the rate of retinol oxidation. Detailed kinetic analysis of *RDH10* and *DHRS9* activities are not currently available. This information will illustrate the potential contributions of these enzymes to the generation of RA in this system. Furthermore, it is important to note that post-transcriptional regulation could play a key role in

determining the activities of these enzymes. Collectively these data suggest that further investigation of the role played by regulation of retinal generation in the generation of RA by immune cells is merited. Future work could assess the influence of modified *DHRS9* expression on the output of RA by CM-treated MoDCs by culturing cells with known differences in *DHRS9* expression in the presence of varying concentrations of retinol and analysing RA in the culture supernatants. This could be developed by analysing the output of RA following knock-down of *DHRS9* expression by MoDCs by siRNA treatment.

'Walk out' cells derived from colonic biopsies displayed a significantly increased ability to induce $\alpha_4\beta_7$ expression on CD4⁺ T cells compared with non-conditioned MoDCs. Interestingly, neither walk out cells nor MoDCs could induce CCR9 expression on CD4⁺ T cells however, indicating that additional factors are required for the induction of this chemokine receptor. The addition of exogenous RA during CD4⁺ T cell activation also did not result in CCR9 expression (data not shown), suggesting that additional factors may be required. In mice, transient TCR stimulation is required in addition to high levels of RA for efficient CCR9 induction on CD4⁺ and CD8⁺ T cells (Ohoka et al., 2011). It is possible that walk out cultures did not meet these TCR stimulation criteria. Alternatively, CCR9 induction is more commonly observed in murine CD8⁺ as opposed to CD4⁺ T cells (Svensson et al., 2002), and induction of CCR9 by human intestinal CD103⁺ DCs in CD patients focussed only on CD8⁺ T cells (Jaensson et al., 2008). The inability of 'walk out' cells to induce CCR9 on CD4⁺ T cells may therefore be related to the nature of the T cell itself. Moreover, CCR9 is associated with homing to the small intestinal mucosa and epithelium (reviewed by Agace, 2006, Sigmundsdottir and Butcher, 2008), and so 'walk out' cells derived from the proximal small intestine may

display an ability to induce CCR9 expression on CD4⁺ and CD8⁺ T cells by delivery additional signal that are currently undefined.

It was recently determined that RA-mediated induction of $\alpha_4\beta_7$ and CCR9 induction in mice requires additional transcription factors such as basic leucine zipper transcription factor, ATF-like (BATF) that direct acetylation of histones and binding of RAR α to gene promoters (Wang et al., 2013). A distinct possibility is therefore that inflamed and non-inflamed IBD CM-treated MoDCs influence expression of such accessory factors that influence the overall level of RAR α signalling and induction of $\alpha_4\beta_7$. The mechanisms by which DC could influence T cell expression of these transcription factors are currently unknown. Preliminary data in this chapter also suggested that this effect is not mediated through modification of *RARA* expression. However, this does not rule out a role for differential post-transcriptional regulation of RAR α in proliferating T cells. Human MoDCs express high levels of *RARA* mRNA with low levels of RAR α protein relative to CD14⁺ monocytes (Fritsche et al., 2000). Inflamed biopsy CM-treated MoDCs could potentially induce a greater level of RAR α protein in allogeneic CD4⁺ T cells than their non-inflamed CM-treated MoDC counterparts.

The presence of detectable RA in IBD but not healthy biopsy-derived CM does not explain differences in $\alpha_4\beta_7$ induction by CM-treated MoDCs. It is however interesting in the light of fact that no differences were detected in RA concentration of intestinal biopsies derived from healthy control and IBD patients in Chapter 4. This may indicate that RA is generated by cells within biopsies during the 6 hour culture period, and to a greater extent in those derived from IBD patients. This may relate to a modified damage response in IBD, and could further implicate RA in intestinal homeostasis.

6:6 Conclusion

In conclusion, this Chapter has demonstrated that differentiation of human MoDCs in the presence of conditioned medium generated using intestinal biopsies from healthy control patients does not recapitulate the slightly enhanced RALDH activity of human intestinal CD103⁺ DCs or significantly enhanced RALDH activity of CD14⁺ MΦ as observed in Chapter 4. However, it reveals a novel mechanism where soluble factors in the inflamed intestinal mucosa of IBD patients confer upon MoDCs an enhanced ability to imprint CD4⁺ T cells with $\alpha_4\beta_7$ expression as compared with factors derived from the non-inflamed mucosa of IBD patients. This is independent of MoDC RALDH activity, as CM from all patient groups suppressed RALDH activity which was partially mediated by PGE₂. The enhanced ability of inflamed IBD CM-treated MoDCs to imprint naive CD4⁺ T cells with $\alpha_4\beta_7$ suggests a novel pathway in the pathogenesis of IBD whereby APCs in the inflamed mucosa have an increased ability to imprint a 'gut-homing' phenotype on responding T cells. This may lead to an increased proportion of the intestinal T cell pool with antigen-specificities biased towards inflamed intestinal mucosa-associated antigen. Lymphocyte migration into the intestinal mucosa is an active area of development in the treatment of IBD (Villablanca et al., 2011a). Understanding how inflammation influences the induction of lymphocyte gut homing by intestinal DCs may provide further insight into the pathogenesis of disease.

Chapter 7: Final discussion and future work

The work presented in this thesis aimed to identify APC subsets capable of RA generation in the distal human intestine, determine whether this production is altered in patients with IBD, and identify how such change might contribute to disease pathogenesis.

The overall picture that emerges from this work is that human CD103⁺ DCs within distal intestinal mucosa have RALDH activity in the steady state associated with *ALDH1A2* expression, and that this activity may be slightly increased during chronic intestinal inflammation in CD and UC patients. RALDH activity is also increased in CD103⁺ DCs in macroscopically non-inflamed intestinal mucosa of active IBD patients, which suggests that this increase may occur prior to the onset of overt inflammation or perhaps that this activity could locally restrain inflammatory processes. Whilst the immunological basis of IBD remains incompletely understood, the hypothesis that loss of RALDH activity in human intestinal CD103⁺ DCs is involved in the pathogenesis of IBD is not supported by these data.

Beyond human intestinal CD103⁺ DCs, CD103⁻ DCs and CD14⁺ MΦ also display RALDH activity. CD14⁺ MΦ within both the macroscopically inflamed and non-inflamed intestinal mucosa of active IBD patients display profoundly increased levels of RALDH activity which is associated with increased expression of *ALDH1A1*. The intestinal CD14⁺ MΦ phenotype with elevated RALDH activity may be recapitulated by culturing CD14⁺ monocytes from healthy controls in GM-CSF. Such CD14⁺ MΦ rapidly generate TNFα in response to LPS stimulation *in vitro*, and so reflect the proposed pro-inflammatory nature of intestinal CD14⁺ MΦ (Kamada et al., 2009). Blocking RARα signalling during this differentiation process fundamentally alters the MΦ phenotype,

with loss of surface CD14, reduced RALDH activity and *ALDH1A1* expression, and reduced ability to produce TNF α following LPS stimulation. *ALDH1A1*-associated RALDH activity was also observed in blood CD14⁺ monocytes from both healthy controls and IBD patients. Taken together, this data supports a model whereby blood CD14⁺ monocytes enter the intestinal mucosa with RALDH activity which, in patients with active IBD, is then selectively upregulated within the mucosa as part of a differentiation process that generates pro-inflammatory CD14⁺ M Φ (see Figure 7.1). An alternative interpretation is that in IBD there is selective recruitment of blood CD14⁺ monocytes with greater levels of RALDH activity, leading to an overall increase in the proportion of intestinal CD14⁺ M Φ with this activity. It would therefore be of interest to determine the chemokine receptor (e.g. CCR2) expression of CD14⁺ blood monocytes with varying levels of RALDH activity in future experiments, to determine whether RALDH activity associates with increased migratory capacity. Within the intestinal mucosa, CD14⁺ M Φ may then contribute to the pathogenesis of IBD through production of a range of pro-inflammatory cytokines including TNF α and IL-23 in response to microbial stimulation (Kamada et al., 2008). At present, it is unknown if the elevated RALDH activity of intestinal CD14⁺ M Φ generates sufficient RA to influence cell differentiation, or if this RALDH activity is the consequence of a process which is generating inflammatory CD14⁺ M Φ in IBD and which is also dependent on RAR α signalling. Further experiments are required to distinguish these possibilities, in particular the differentiation of blood CD14⁺ monocytes in the presence of DEAB to inhibit RALDH activity throughout GM-CSF-mediated differentiation *in vitro*. Alternatively, *ALDH1A1* expression could be suppressed by siRNA to specifically limit the influence of this RA-generating enzyme.

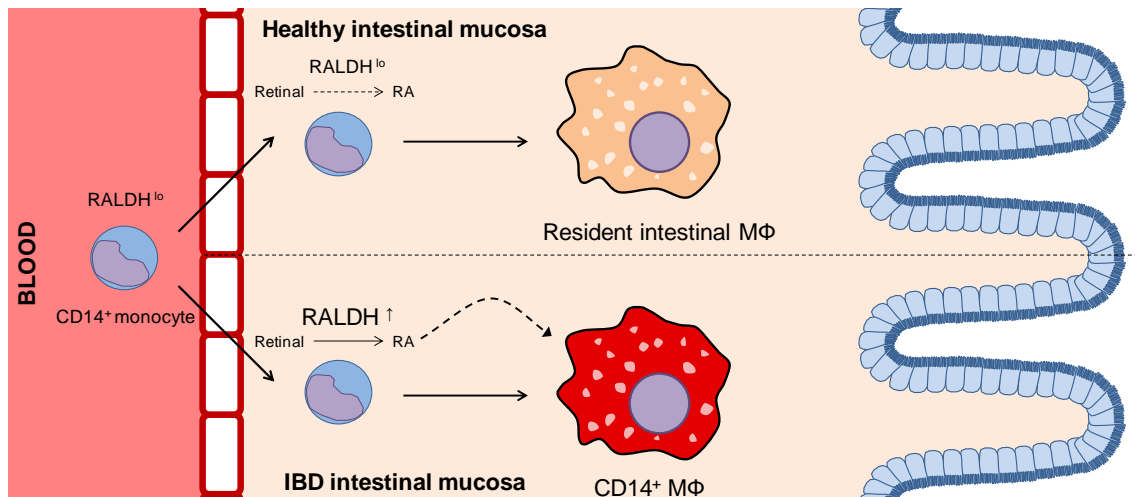


Figure 7.1: Proposed role of RALDH activity in promoting differentiation of human CD14⁺ monocytes into CD14⁺ MΦ in IBD. Circulating blood CD14⁺ monocytes have low levels of RALDH activity. Upon entering the intestinal mucosa in healthy controls (upper panel), RALDH activity remains basal and monocytes differentiate into the resident intestinal CD14^{-/lo} MΦ phenotype. In contrast, CD14⁺ monocytes enter the intestinal mucosa of IBD patients (lower panel) and upregulate RALDH activity. RARα signalling promotes retention of CD14 expression and differentiation into a pro-inflammatory CD14⁺ MΦ phenotype.

The preliminary analysis in this thesis indicates that the overall levels of RA are not significantly different in the mucosa of CD patients, which suggests that changes in RALDH activity within APC populations are not sufficient to affect the overall levels of RA present within the mucosa. However, the RALDH activity of stromal and epithelial cells is slightly reduced in IBD, consistent with reduced *ALDH1A1* expression in CD epithelial cells (Iliev et al., 2009). This may indicate that varying levels of RALDH activity in different cell compartments maintain an overall homeostasis of RA levels. This does not however rule out significant local variations in RA production and signalling at the cellular level. RA is regarded as an important morphogen capable of diffusing through tissues and influencing gene expression in a concentration-dependent

manner (reviewed by Niederreither and Dolle, 2008). Thus local RA concentration in a particular microanatomical location may be more relevant than overall tissue levels.

The data presented in Chapters 3 and 6 demonstrate that the Aldefluor assay provides a clear indication of the level of RALDH activity present within human DCs. However, it is also apparent that the loss of RALDH activity associated with differentiation of MoDCs in the presence of human intestinal biopsy-derived conditioned medium does not reduce the ability of these cells to induce RAR α -dependent expression of $\alpha_4\beta_7$ by allogeneic CD4⁺ T cells. Therefore, the presence of RALDH activity alone is not necessarily sufficient to generate high enough levels of RA to influence neighbouring cells. For this reason, care is required when interpreting the presence of RALDH activity in cells. The overall output of RA may be determined by the ability of cells to generate retinal from retinol by the activity of microsomal retinol dehydrogenase enzymes including RDH10. Whilst the expression of such enzymes may be confirmed by real-time PCR and protein analysis, an equivalent Aldefluor assay that directly monitors the ability of cells to oxidise retinol to retinal is not currently available. A further important consideration is the ability of cells to oxidise RA to inactive metabolites through the activity of CYP26 enzymes. In development, spatial and temporal regulation of CYP26 enzymes controls RA responsiveness (Hernandez et al., 2007). A study published late in the preparation of this thesis identified a weak association between a polymorphism in *CYP26B1* and CD, possibly leading to reduced RA catabolism and therefore increased RA signalling (Fransén et al., 2013). The overall output of RA is therefore likely to involve a complex range of factors, of which RALDH activity is only one component.

The trend towards increased RALDH activity in human intestinal CD103⁺ DCs contrasts with data from the TNF Δ ARE and T cell transfer models of colitis in which RALDH

activity of MLN CD103⁺ DCs is reduced during inflammation (Laffont et al., 2010, Collins et al., 2011). This may result from the fact that tissue used in this project derived predominantly from the distal human intestine, i.e. the colon and TI, whilst MLN CD103⁺ DCs are likely to have drained from the SI mucosa where CD103⁺ DCs display the greatest levels of RALDH activity (Villablanca et al., 2011b, Schulz et al., 2009). CD103⁺ DCs within proximal human intestine e.g. the jejunum may display greater levels of RALDH activity which may play a role in diseases such as Coeliac disease which affect the small intestinal mucosa (reviewed by Marsh, 1992). However, CD103⁺ DCs in the inflamed TI mucosa of TNFΔARE mice also express significantly reduced levels of *Aldh1a2* compared with WT mice (Collins et al., 2011), suggesting that if the loss of RALDH activity in mice and humans in intestinal DCs during inflammation was shared between species, loss of RALDH activity should have been apparent in the present study. Whilst the need to group TI and colonic samples due to the low number of samples available during the study is a clear limitation, there is little difference in the RALDH activity of murine colonic and TI DCs (Villablanca et al., 2011b). As discussed above however, it is important to note that RALDH activity alone may not completely determine the overall RA output and so a range of additional factors including retinol oxidation and RA metabolism need to be considered in future work. A report published late during the preparation of this thesis identified increased Aldefluor activity in CD11c⁺ cells in the colonic mucosa of DSS-treated mice (Mielke et al., 2013). As the CD11c⁺ compartment is likely to contain both DCs and MΦ populations, this supports the conclusion that RALDH activity in murine intestinal APCs is enhanced under certain inflammatory conditions, as in the present study.

Beyond a potential role in the differentiation of CD14⁺ MΦ within the intestinal mucosa of IBD patients, the effect of increased RA production by CD14⁺ MΦ on neighbouring

cells and how this might affect disease pathogenesis remains unclear. It may exert protective roles including stimulation of local $\gamma\delta$ T cells and innate lymphoid cells to produce IL-22 as observed in mice (Mielke et al., 2013). IL-22 induces production of the antimicrobial peptides RegIII γ and RegIII β in murine colonic epithelial cells during acute bacterial infection (Zheng et al., 2008), and plays a key role in STAT3-mediated epithelial wound healing during DSS colitis (Pickert et al., 2009). IL-22 is not detectable in the steady state human intestinal mucosa, but is observed in UC and at greater levels of CD patients (Andoh et al., 2005). Such a protective role for RA generation may be consistent with the presence of enhanced RALDH activity in CD14⁺ M Φ in the non-inflamed intestinal mucosa of active IBD patients. This may be part of a process in which inflammation is being actively restrained within these mucosal areas. The extent to which the macroscopically non-inflamed mucosa of such patients matches that of patients in remission is unclear, and it would be of interest to compare the RALDH activity of CD14⁺ M Φ in these different tissues. RA produced by CD14⁺ M Φ may also exert different functions in non-inflamed and inflamed tissue, depending on the presence of additional cytokines. This is observed in mice where RA in the presence of IL-15 supports IL-12p70 production by intestinal DCs and reduces their ability to induce Foxp3⁺ T_{REG} (DePaolo et al., 2011).

The model described above proposes that the failure of blood CD14⁺ monocytes entering the steady state intestinal mucosa to upregulate RALDH activity leads to differentiation into CD14⁻ resident M Φ . Currently, human intestinal resident M Φ are identified as CD14⁻ (Smythies et al., 2005) or alternatively CD14^{lo} (Bain et al., 2013), depending on techniques used to isolate and identify these cells. Understanding the role of RALDH activity in the differentiation of blood CD14⁺ monocytes into different M Φ phenotypes will require a better mechanistic understanding of this process.

Furthermore, the *in vitro* system of CD14⁺ MΦ differentiation outlined in Chapter 5 is limited in that it is removed from the physiological setting. These issues may be addressed by the development of a new model system in which cell trace (e.g. CFSE) labelled blood CD14⁺ monocytes are cultured in the presence of autologous intestinal biopsy-derived LPCs from healthy controls and IBD patients. This would allow the differentiation of these cells to be monitored in a more physiological setting over several days, as well as identifying the point at which RALDH activity becomes upregulated during this process. The influence of factors such as GM-CSF and RA signalling on this differentiation process could therefore be monitored with the use of specific monoclonal antibodies and inhibitors.

Ly6C^{hi} monocytes entering acutely inflamed intestinal mucosa during *Toxoplasmosis gondii* infection of mice produce PGE₂ which suppresses neutrophil activation and therefore prevents excessive tissue damage (Grainger et al., 2013). Human blood CD14⁺ monocytes also produce PGE₂ following stimulation with LPS (Grainger et al., 2013). It is likely therefore that human blood CD14⁺ monocytes entering the intestinal mucosa possess the ability to generate PGE₂. Whether CD14⁺ MΦ continue to produce PGE₂ in IBD and how this influences neighbouring cells including neutrophils remains to be determined. PGE₂ suppresses RALDH activity of human MoDCs through suppression of *ALDH1A2* expression (Stock et al., 2011). As observed in Chapter 6, PGE₂ is an important factor in the suppression of *ALDH1A2*-associated RALDH activity in human MoDCs by intestinal biopsy-conditioned medium. Whether the elevation of RALDH activity in human intestinal CD14⁺ MΦ in IBD is related to alterations in PGE₂ production or signalling by these cells within the diseased mucosa remains to be determined.

The mechanism behind the increased ability of human MoDCs grown in the presence of inflamed intestinal biopsy-conditioned medium to induce $\alpha_4\beta_7$ on proliferating CD4⁺ T cells remains undefined. It is possible that this effect is mediated by unknown factors acting in synergy with RA. If DCs within inflamed intestinal mucosa are able to imprint an increased level of $\alpha_4\beta_7$ this may result in a population of effector T cells specific for antigen associated with the inflamed intestinal mucosa with a selective advantage in their ability to home into the gut mucosa through interaction with endothelial MAdCAM-1. Identifying factors acting in synergy with RA to induce $\alpha_4\beta_7$ in the context of intestinal inflammation may provide an additional therapeutic target by which the imprinting of T cells with inflammatory potential is selectively inhibited, whilst that of other T cells associated with antigen derived from non-inflamed areas may remain unaffected.

Understanding phenotype differences between blood CD14⁺ monocytes in healthy controls and IBD patients is likely to be a key area for future research. In particular, monocyte-derived M Φ in CD produce significantly reduced levels of pro-inflammatory cytokines in response to microbial stimulation in CD patients (Smith et al., 2009, Sewell et al., 2012), which may result in defective innate clearance of encroaching bacteria within the intestinal mucosa. The present study identified significantly increased expression of the oncogene *MN1* in blood CD14⁺ monocytes in IBD patients compared with healthy controls. The differentiation of human CD34⁺ bone marrow HSCs to either CD14⁺ monocytes or CD15⁺ granulocytes associates with a significant downregulation of *MN1* (Kandilci and Grosveld, 2009). This indicates that monocytes circulate in IBD in a more immature state of differentiation, potentially through modulations in the haematopoiesis program within the bone marrow in response to inflammatory signals as observed in mice (reviewed by (King and Goodell, 2011)). Consistent with this view, it is well established that inflammation associates with increased production and release

of immature monocytes from the bone marrow into circulation in humans (Meuret et al., 1974). Moreover, the T cell transfer model of colitis associates with a significant expansion in the number of granulocyte/macrophage progenitors (GMPs) in the bone marrow, spleen, and also within the inflamed colonic mucosa (Griseri et al., 2012), consistent with peripheral circulation of immature progenitor cell populations. Forced expression of *MN1* in primary human HSCs inhibits differentiation into monocytes and granulocytes *in vitro* (Kandilci and Grosveld, 2009). Following confirmation of the overexpression of *MN1* in blood CD14⁺ monocytes in IBD patients in a larger study, it will be of interest to determine the effect of suppressing *MN1* through siRNA on the ability of monocytes to produce pro-inflammatory cytokines in response to microbial stimulation. This could also be applied to monocytes used in the *ex vivo* intestinal differentiation model outlined above to determine if it leads to modified induction of RALDH activity and differentiation into distinct MΦ subsets. Finally, it would be of interest to determine whether *MN1* expression is modified in blood CD14⁺ monocytes of first degree relatives of IBD patients which possess an 'at risk' phenotype for developing disease (reviewed by Hedin et al., 2012). This may help to determine whether alterations in haematopoiesis predispose patients to the onset of disease, and whether these changes are an important early component of disease pathogenesis.

Overall, this thesis has demonstrated that the loss of RALDH activity of CD103⁺ DCs in IBD is unlikely to contribute to the pathogenesis of IBD. However, it implicates RALDH in the differentiation of CD14⁺ MΦ in the intestinal mucosa of IBD patients. Modulating this differentiation so as to favour the non-inflammatory resident MΦ phenotype may provide a novel therapeutic approach in the treatment of IBD.

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Appendix: Summary patient data

Chapter 4

	HC (%)	CD (%)	UC (%)
Number of subjects	32	34	7
Male	21 (66)	18 (53)	3 (43)
Female	11 (34)	16 (47)	4 (57)
Average age (years)	57.6	25.9	38.3
Tissue samples	32	40	8
Colon	31 (96.9)	31 (78)	7 (88)
TI	1 (3.1)	9 (22)	1 (12)
Active disease			
Number of subjects		27	4
Number of samples		33	5
Colon (inflamed)		17	4
Colon (non-inflamed)		7	0
TI (inflamed)		6	0
TI (non-inflamed)		3	1
Inactive disease			
Number of subjects		7	3
Number of samples		7	3
Colon		7	3
TI		0	0
Medication			
AZA	N.D.	14 (41)	1 (14)
Anti TNF α	N.D.	9 (26)	0 (0)
MTX	N.D.	2 (6)	1 (14)
5-asa	N.D.	4 (12)	2 (29)
Steroids	N.D.	7 (21)	2 (29)
6-MP	N.D.	4 (12)	0 (0)

Several IBD patients were receiving combination therapies at the time of tissue collection. N.D. = no data, AZA = azathioprine, anti-TNF α includes both infliximab and adalimumab, MTX = methotrexate, 5-asa = 5 aminosalicylic acid (mesalazine), 6-MP = 6 mercaptopurine.

Chapter 5

	HC (%)	CD (%)	UC (%)
Number of subjects	7	10	4
Male	4 (57)	4 (40)	1
Female	3 (43)	6 (60)	3
Average age (years)	31.4	20.9	
Active disease			
Number of samples		6	3
Inactive disease			
Number of samples		4	1
Medication			
AZA		8 (80)	3 (75)
Anti TNF α		3 (30)	0 (0)
MTX		0 (0)	1 (25)
5-asa		1 (10)	1 (25)
Steroids		0 (0)	0 (0)
6-MP		0 (0)	0 (0)

Samples used in Chapter 5 were exclusively for the analysis of blood CD14⁺ monocytes.

Chapter 6

	HC (%)	CD (%)	UC (%)
Number of subjects	10	10	7
Male	8 (80)	7 (70)	4 (57)
Female	2 (20)	3 (30)	3 (43)
Average age (years)	52.9	30.4	31.0
Tissue samples	12	16	9
Colon	10 (83)	13 (81)	8 (89)
TI	2 (17)	3 (19)	1 (11)
Active disease			
Number of subjects		9	5
Number of samples		15	7
Colon (inflamed)		7	5
Colon (non-inflamed)		5	1
TI (inflamed)		2	0
TI (non-inflamed)		1	1
Inactive disease			
Number of subjects		1	2
Number of samples		1	2
Colon		1	2
TI		0	0
Medication			
AZA		2 (20)	3 (43)
Anti TNF α		1 (10)	1 (14)
MTX		0 (0)	0 (0)
5-asa		1 (10)	5 (71)
Steroids		3 (30)	2 (29)
6-MP		0 (0)	0 (0)